THE ROLE OF TRANSLATION INITIATION REGULATING KINASES, *LK6* AND *TOR*, ON NOCICEPTOR DEVELOPMENT AND FUNCTION IN *DROSOPHILA MELANOGASTER*

A Thesis by
HALEY ALEXIS MCGUIRT

Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2019
Department of Biology

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Abstract

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The cellular and molecular mechanisms responsible for nociception have been studied extensively, but remain incompletely understood. Dysregulation of nociceptor plasticity causing long lasting changes in sensitivity is likely a primary cause of chronic pain. Chronic pain currently affects over 100 million adults in the United States, while the Institute of Medicine reports that chronic pain costs the US over $500 billion dollars annually due to expenses associated with healthcare treatments and lost productivity. Effective treatments for chronic pain are lacking due to our incomplete understanding of the complex mechanisms that underly the development and persistence of chronic pain. To improve current chronic pain therapies, it is important to characterize the signaling pathways responsible for nociceptor sensitivity. The goal of this study was to characterize the role of specific genes that encode components of signaling pathways that regulate unique aspects of nociceptor sensitivity. The model organism *Drosophila melanogaster* was used for this study due to the availability of a quantifiable response to noxious stimuli and the powerful tools available for

genetic manipulation. This project focused on characterizing the involvement of two kinases, Lk6 and Target of Rapamycin (Tor), that are known to positively regulate the rate of translation initiation through interaction with eukaryotic initiation factor 4E (eIF4E), in the regulation of nociceptor sensitivity. Expression levels of *Lk6* and *Tor* in the nociceptors of *Drosophila* larvae were altered using the GAL4/UAS-RNAi system. The effects of altered gene expression levels on baseline nociceptor sensitivity, dendritic morphology, and the development of injury induced hypersensitization were quantified. The results suggest nociceptor-specific knockdown of *Lk6* played an important role in the development of thermal nociceptor sensitization following UV-induced tissue injury, while playing no role in baseline nociceptor sensitivity. *Lk6* likely controls the translation of mRNAs known to be involved in nociceptor plasticity or signal transduction. Nociceptor-specific knockdown of *Tor* showed phenotypes that suggested the involvement of the *Tor* signaling pathway in baseline nociceptor sensitivity, and it is hypothesized that *Tor* would also play a role in the translation of mRNAs known to be involved in nociceptor plasticity.

Acknowledgements

I would like to first and foremost thank my mentor, Dr. Andrew Bellemer, Andy, for the development of my research project. Without your knowledge of and dedication to the study of translational control of nociception my project and master's degree would not have been possible. Thank you for your constant support and patience. Thank you to Dr. Mark Venable for supporting me throughout the duration of my educational career at Appalachian State as both a professor and mentor. Thank you to Dr. Chishimba Mowa for not only supporting my career in biology through both my undergraduate and master's degrees, but for always being conscious of my well-being. A huge thank you to my peers: Katherine Hoffman, for showing my generation of graduate students the way; Adam Willits, for the constant deadline reminders and confocal microscopy skills; Gita Gajjar, for the honest friendship and laughs; MacKinsey Johnson, Katie Hahn, and Elsie Rodriquez for understanding the day to day struggles that came with this journey. Thank you to the Office of Student Research for the funding and numerous opportunities to share my work with the Appalachian Community. Thank you to Cratis D. Williams Graduate School and the Department of Biology for making everything possible. And lastly, thank you to Appalachian State University, the administration, staff, and professors for the best six years of my life; there is no educational community quite like ours. This town and university will always be a home away from home.

Dedication

This thesis is dedicated to my family and friends.
nothing would be possible without you.

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Introduction

Chronic Pain

Chronic pain is defined as a pain that persists longer than 90 days or after an injury has healed. This medical issue has become a major epidemic across the globe. It is estimated that over 100 million Americans experience chronic pain each day, while globally, it is estimated that the number of those affected reaches 1.5 billion. Chronic pain is a major health problem worldwide, with an economic burden of up to $635 billion a year in health care cost and lost productivity in the United States alone. [1-3] A central theme outlined in a 2011 Institute of Medicine report was that despite the fact that the treatment of chronic pain is extremely costly, outcomes continue to remain relatively poor. [2] A wide variety of treatments are being used to treat chronic pain including, antidepressant, local anesthetics, opioid analgesics, and tramadol. 40-60% of patients being treated for chronic pain report that the current therapies are ineffective for long term relief. [4]

Evidence from recent studies support the notion that the use of opioids for the treatment of chronic pain only has short-term efficacy and should not be used as a long-term treatment option due to side effects including addiction. [5] In 2016, the Centers for Disease Control (CDC) published guidelines on the use of opioids for the treatment of chronic pain as a response to the abundance of opioids being prescribed in combination with the alarming rise in opioid use disorders and deaths in the United States. Opioid prescriptions rose by 7.3% during the years 2007-2012. During this five-year span, 259 million prescriptions were written, which equates to enough prescriptions for every adult in the United States to have one. [6-7] Recent studies have begun to illuminate cellular targets of nociception that could result in a variety of new therapies to treat chronic pain. The development of new treatments

that can target nociception more directly may provide safer and more effective alternatives to opioids. [8]

Nociception

Nociception is the nervous system's response to harmful or potentially harmful, noxious, stimuli. Nociceptive pathways can be activated by intense chemical, mechanical, or thermal stimulation of sensory neuron cells called nociceptors. Nociceptors are neurons that are associated with free nerve endings that detect noxious thermal, mechanical, or chemical stimuli and are found in the skin and internal surfaces of the body. [9] The concentration of nociceptors varies, but they are found in greatest concentration on the skin. Nociceptors can be divided into two major classes: Aδ fibers and C fibers. Aδ fibers can be characterized as medium diameter myelinated afferents that mediate acute, well localized, "first" or fast pain. C fibers convey poorly localized "second" or slow pain and are characterized as small diameter unmyelinated afferents. [10] These nociceptors act as a protective feature as they are only activated when a stimulus has the potential to cause tissue damage. Nociceptors are able to be selectively activated due to the fact that they have activation thresholds, meaning that they require a minimum intensity of stimulation before they can trigger a signal that will be passed along the axon of the neuron to the spinal cord. [10] This means that they detect stimuli with the potential to cause tissue damage, but not innocuous stimuli.

Sensory neurons extend into the spinal cord through the dorsal horn of the spinal cord. The dorsal root ganglion (DRG) houses the cell bodies of the sensory neurons. The DRG functions to transduce and modulate sensory information moving into the spinal cord. [11] Once the signal enters the spinal cord, the sensory neuron sends it through an

interneuron, or multiple interneurons, in the spinal cord to higher order brain regions for processing. [12-14] This process of nociception triggers a variety of physiological and behavioral responses that typically result in the subjective experience of pain coupled with behavioral reactions, such as the withdraw reflex. (Figure 1) Nociception is essential to an organism's protection and survival. However, when the nociceptive pathway malfunctions, an organism's ability to appropriately detect, react to, and recover from injury can be altered resulting in chronic pain. [14-15]

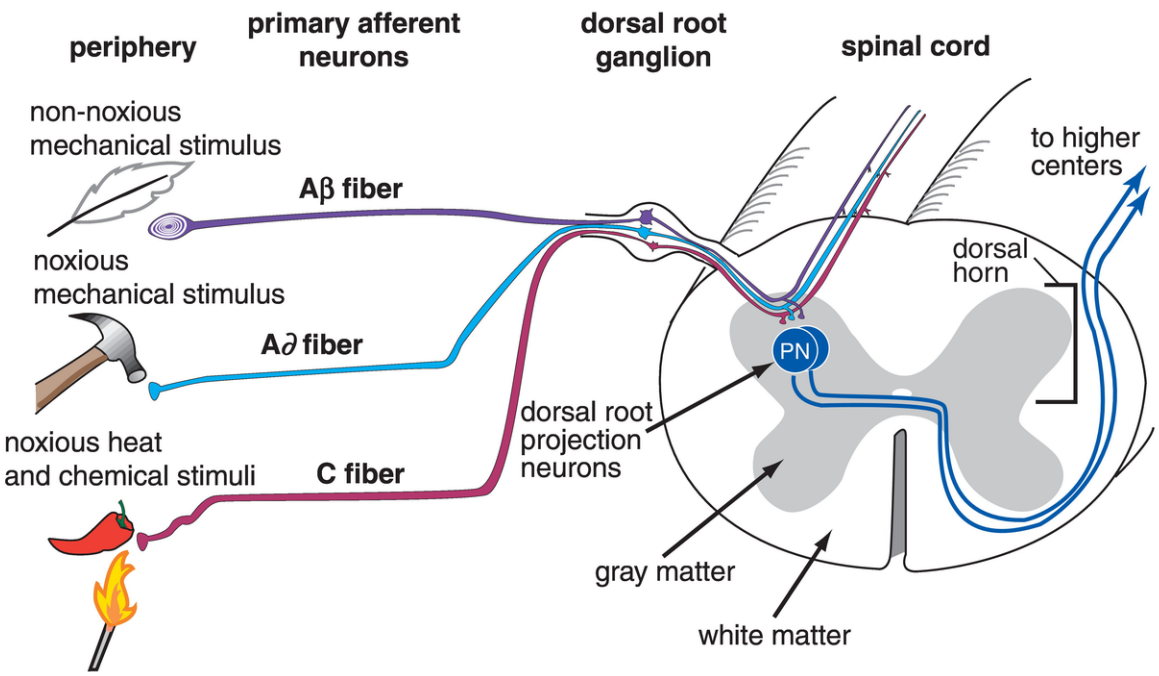

Figure 1. Depiction of the activation of nociceptive nerve fibers. Detection of a noxious stimulus occurs at the peripheral terminals of primary afferent neurons and leads to generation of action potentials that propagate along the axon to the central terminals. Aβ fibers respond only to non-noxious stimuli, Aδ fibers respond to noxious mechanical stimuli and subnoxious thermal stimuli, and C fibers respond only to noxious mechanical, heat, and chemical stimuli. [16]

Nociceptor Plasticity

Nociceptive pathways are activated in response to noxious of harmful stimuli. The acute sensation of pain produced by nociception serves as an adaptive and protective mechanism to detect, localize, and prevent further tissue damage. However, when pain persist in conditions of chronic pain, after a normal healing period has occurred (1-3 months), a maladaptive response has developed and is no longer beneficial to the organism's protection or survival. In states of chronic pain, pain arises in the absence of noxious stimuli and maybe stimulated by normally innocuous stimuli due to a reduced threshold for the activation of nociceptors (allodynia). (Figure 2) Chronic pain conditions can also result in an exaggerated and prolonged response to noxious stimuli (primary hyperalgesia), and spread beyond the site of injury (secondary hyperalgesia). (Figure 3) [20] Neural plasticity, or changes within the structure and physiology of neurons or availability of the proteins required for nociception within primary afferent nociceptors can lead to these modes of long lasting hypersensitization. [14, 17-18]

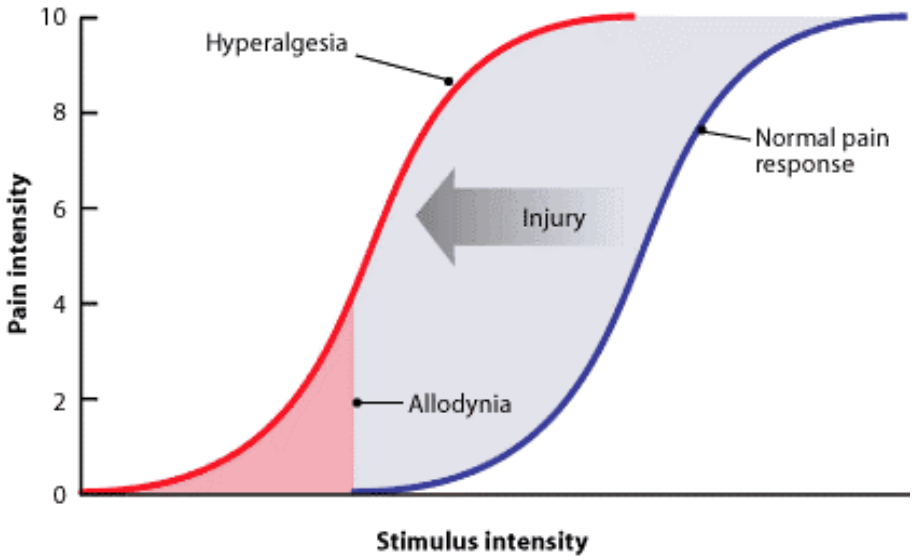

Figure 2. Depiction of allodynia. In states of chronic pain, pain arises in the absence of noxious stimuli and maybe be stimulated by normally innocuous stimuli (allodynia) due to a reduced threshold for the activation of nociceptors. [19]

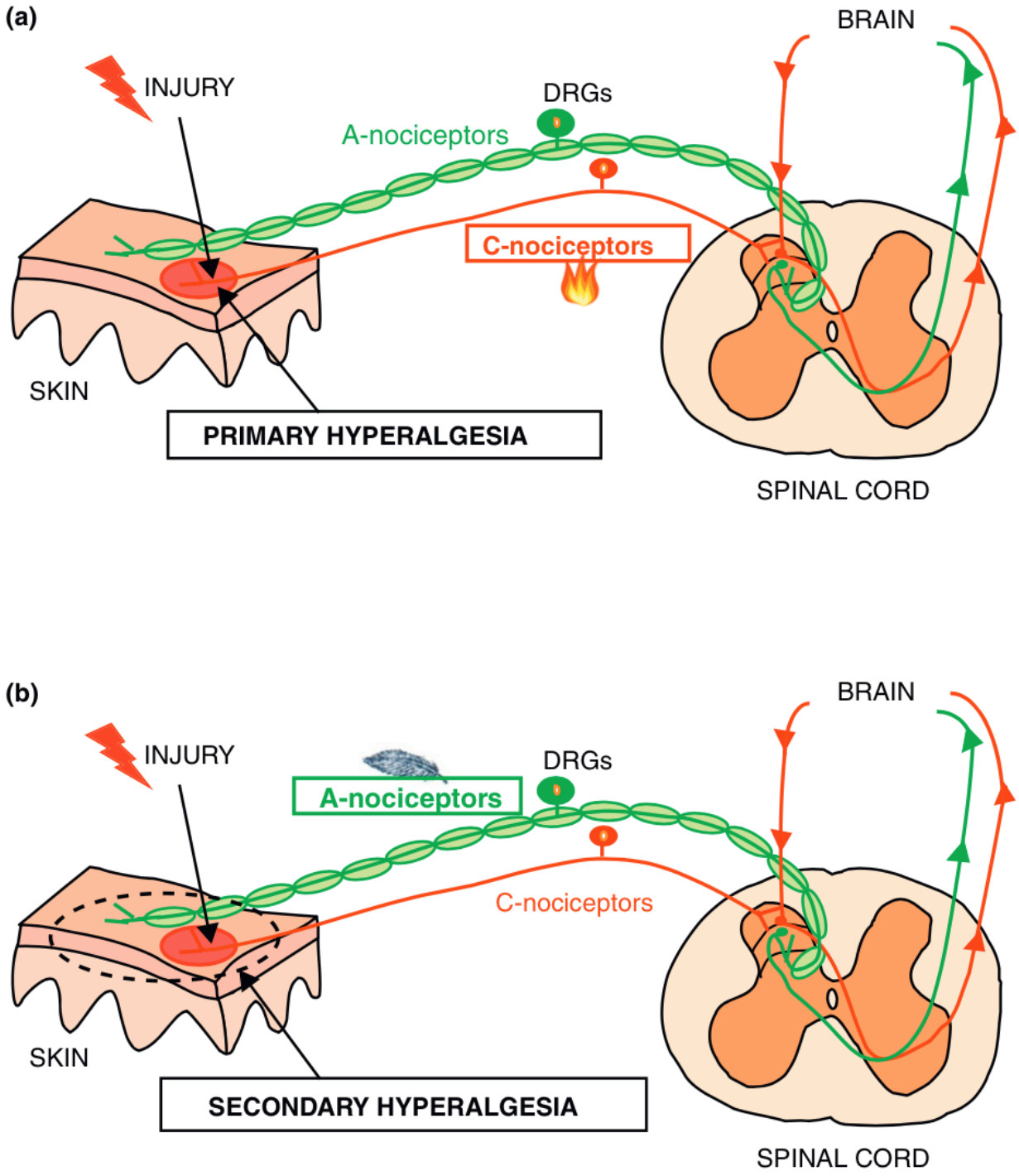

Figure 3. Depiction of primary (a) and secondary (b) hyperalgesia. Chronic pain conditions can result in an exaggerate and prolonged response to noxious stimuli (primary hyperalgesia), and spread beyond the site of injury (secondary hyperalgesia). [20]

Neural plasticity within both the peripheral nervous system (PNS) and the central nervous system (CNS) is a major mechanism that leads to the development of chronic pain. [4] Chronic pain is believed to develop through neural plasticity and consequently changes in the excitability of peripheral nociceptive neurons, but the precise mechanisms controlling these changes have not been fully elucidated. It has been hypothesized that molecules which regulate translation play a major role in controlling the expression of genes necessary for the transition of nociceptors from standard activation states to states of hypersensitivity (allodynia or hyperalgesia). [17]

Translational regulators, such as mammalian target of rapamycin (mTOR) kinase and mitogen activated protein kinase (MAPK) interacting kinases 1 and 2 (MNK1 and MNK2) are involved in translation regulation pathways that play a role in neural plasticity and changes in nociceptor excitability. [21] Recent studies have shown that mTOR and its downstream effectors may be important in the development of chronic pain stemming from cancer, neuropathy, and inflammation. [22-24] The mTOR kinase is a master regulator of protein synthesis, and has been shown to be involved in several neural functions including synaptic plasticity. mTOR could, therefore, be a novel pharmacological target for the management of chronic pain. [25] It has also been shown that phosphorylation of the main mRNA translational regulator, eukaryotic initiation factor (eIF) 4E (eIF4E), is an additional mechanism that is critical for the regulation of the changes in nociceptor plasticity that drive the development of chronic pain. Targeting, MNK, the kinase responsible for the phosphorylation of eIF4E is an important potential therapeutic target for the treatment of chronic pain as well. [26]

Translation of Nascent Proteins Leads to Neural Plasticity

Translational control within neurons contributes to the development of the peripheral and central nervous systems, is involved in axonal regeneration following injury, and allows for neuronal plasticity. Neuronal plasticity is defined as a neuron's ability to adapt to environmental changes via intracellular and extracellular signaling pathways through many mechanisms, including changes in gene expression and thus protein abundance. The synthesis of new proteins has been observed in cases of injury, suggesting that upregulation of specific proteins is a critical factor for neuronal plasticity. [27] Gene expression is regulated at multiple levels, including the translation of mRNAs into proteins. Compared to transcriptional regulation, translational control of mRNA allows for more rapid changes in the cellular concentration of the encoded proteins and thus can be used to maintain homeostasis or induce more permanent changes such as strengthening or weakening of neural synapses. [28-29]

Both mTOR and MNK are known to act upstream of translation initiation to positively regulate translation. Specifically, in the context of nociception, it is hypothesized that these translational regulators may act to regulate the expression of genes that encode proteins that are necessary for nociceptor hypersensitization. Evidence from many studies suggest that local translation, for example translation that occurs within the axon, independent of the cell body, is crucial for the establishment and maintenance of chronic pain states. Local translation of mRNA within an axon would be advantageous as it would allow for a more rapid and localized response to environment changes or insults. [17] Controlling the abundance of proteins necessary for nociception and the transition to states of hypersensitization, perhaps through the inhibition of translation regulators, could become a

central strategy in the development of pharmaceuticals used to replace opioids in the treatment of chronic pain. [28-30]

The Basics of Translation

Translation can be separated into three main steps: initiation, elongation, and termination. Initiation is considered the most highly regulated, rate-limiting step and requires proper assembly of the eIF4F complex on the mRNA before ribosome recruitment and subsequent steps of initiation can proceed. Most eukaryotic mRNAs are translated in a cap-dependent manner with the exception of those that are translated via internal ribosome entry sites (IRES). Cap-dependent translation begins when the eIF4F complex assembles and binds to the 5' cap of the mRNA strand. The 5' cap is a 7-methylguanosine attached to the first nucleotide of the 5' untranslated region (UTR) of the mRNA. Following the assembly of the eIF4F complex, the ribosomal subunits will be recruited, and scanning of the 5' UTR for the start codon will proceed. [22]

The eIF4F complex is comprised of three key eIFs: eIF4E, eIF4A, and eIF4G. eIF4E, the most highly regulated member of the eIF4F complex, is responsible for identifying and binding to the 5' cap. eIF4G, the scaffolding protein, interacts directly with eIF4E, eIF4A, eIF3 and the poly-A binding protein (PABP). Interaction of eIF4G with PABP allows for circularization of the mRNA and shuffling of ribosomes from the termination codon to back to the 5' cap. eIF4A and other helicases, such as Ded1 and DHX29, are responsible for unwinding the secondary structure of the 5' UTR allowing for effective ribosomal scanning for the initiation codon. [22]

Assembly of the eIF4F complex is followed by recruitment of the 43S preinitiation complex (PIC) through direct binding to the 40S ribosomal subunit. The scanning process then proceeds in a 5' to 3' direction until the PIC, loaded with a met-tRNA, recognizes the start codon (AUG in most cases). After start codon recognition, the 60S ribosomal subunit is recruited, forming the 80S ribosomal subunit which marks the beginning of the elongation phase of translation. [22] (Figure 4)

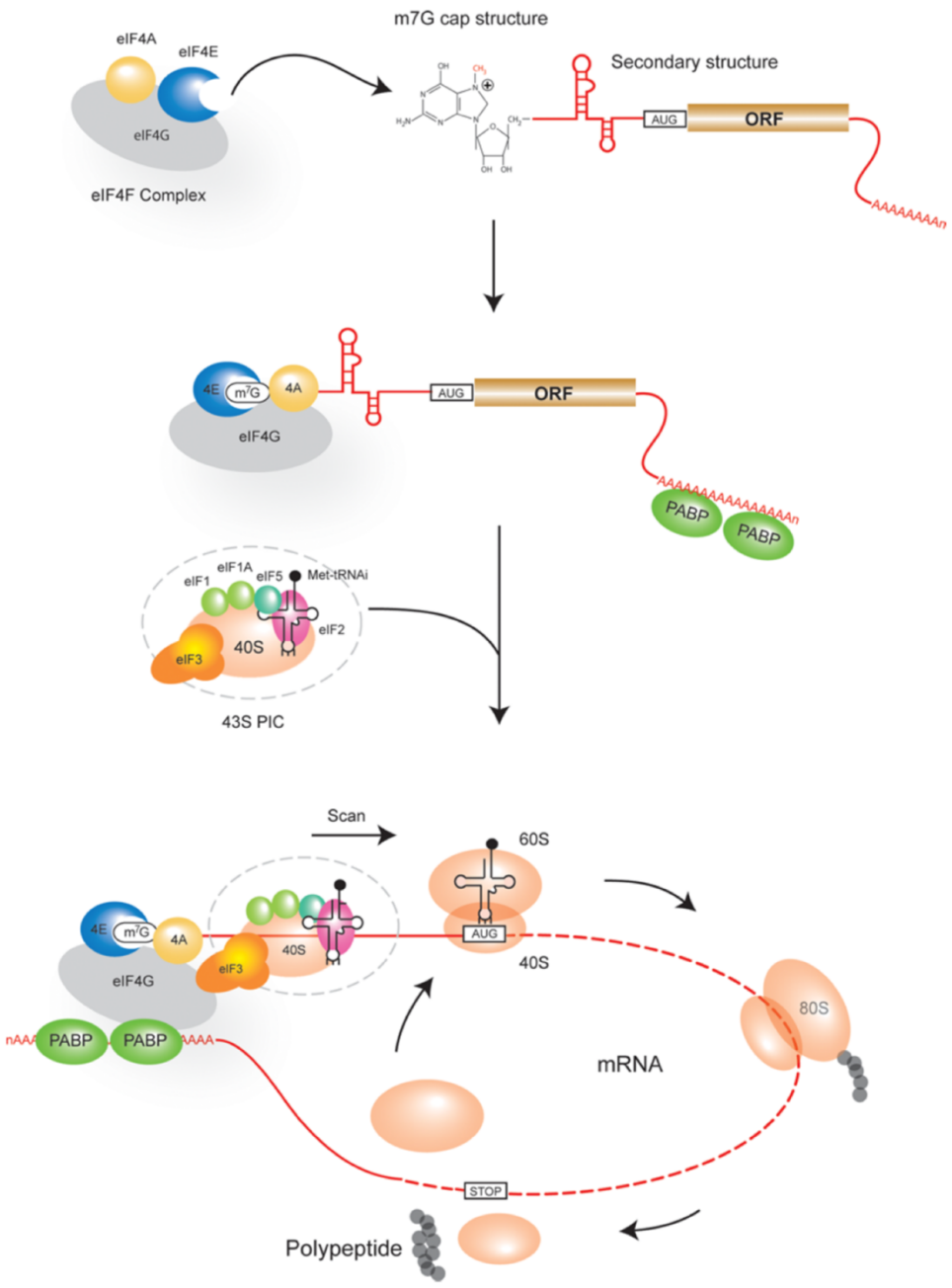

Figure 4. Representation of cap-dependent translation. eIF4F complex binds to the 5' cap via eIF4E. eIF4G binds to eIF4A and eIF3 recruiting the 43S PIC. eIF4A unwinds the 5' UTR secondary structure while the PIC scans until it encounters the initiation codon. The 60S subunit joins and elongation commences. eIF4G interacts with PABP allowing for mRNA circularization. [22]

eIF4E Plays a Major Role in the Regulation of Translation Rates

Evidence has shown that chronic pain states are accompanied by major changes in translational activity and thus the abundance of proteins involved in nociception. Major changes in the function and availability of key translational machinery and molecules required for normal nociception are seen in instances of tissue damage followed by chronic pain. [31] These changes have been directly linked to increases in eIF4F complex formation and nascent protein synthesis. eIF4E is the rate limiting factor for eIF4F assembly as it is found in least abundance. The importance of eIF4E regulation in the assembly of the eIF4F complex and translation initiation suggest that eIF4E could be a promising therapeutic target to inhibit aberrant pain plasticity. [30]

Expression levels of eIF4E are the lowest among all of the eIFs that makeup the eIF4F complex, thus making formation of eIF4F complex and correspondingly translation initiation reliant upon the availability and activity of eIF4E. Some mRNAs are more sensitive to the accessibility of eIF4E than others. Translation rates of eIF4E-sensitive mRNAs are preferentially stimulated by increased eIF4E activity. Characteristics of these mRNAs include mRNAs that possess a complex secondary structure, a long guanosine/cytosine rich 5' UTRs, cis-regulatory elements, TOPS (5' terminal oligopyrimidine tracts), or CERTS (cytosine enriched regulators of translation). eIF4E-sensitive mRNAs do not make up the mass majority of mRNAs involved in general translation, however they do include vital mRNAs critical for cell proliferation, growth, and immune responses. [28] eIF4E integrates cellular signals and is regulated by two major signaling pathways: MNK and mTOR. Inhibition of MNK and mTOR signaling alleviates the development of nociceptor hypersensitivity in a variety of pain models. Because MNK and mTOR converge on eIF4E to

control the rate of cap-dependent translation, it can be suggested that eIF4E might play a role in the sensitization of pain circuits by regulating the translation of specific mRNAs. [28-32]

Depletion of eIF4E and associated proteins reduces cap-dependent translation. The reduction in translation due to loss of eIF4E can be mitigated when purified eIF4E is re-introduced *in vitro*. [33] Many mechanisms regulate the availability of eIF4E including amplification of the eIF4E gene, post-transcriptional modifications such a phosphorylation, and interactions with inhibitory proteins such as 4E-binding proteins (4E-BPs). [34] The physiological significance of eIF4E phosphorylation was studied using mice lacking eIF4E phosphorylation through a knock-in mutation of serine209 to alanine (*eIF4E*^{S209A}). These mice displayed greatly reduced mechanical and thermal hypersensitivity as well as diminished hyperalgesic priming (prolonged inflammatory mediator-induced hyperalgesia) in response to intraplantar administration of proinflammatory mediators: IL-6, NGF, and carrageenan. This indicates that reduced phosphorylation of eIF4E results in decreased nociceptor sensitivity caused by inflammatory mediators. [31]

A dominant mechanism that regulates the activity of eI4FE is through interaction with a family of translational suppressor proteins called 4E-binding proteins (4E-BPs). Binding of 4E-BPs to eIF4E is highly regulated by their status of phosphorylation. Phosphorylation of specific serines and threonines on the 4E-BP molecule modulates the affinity of 4E-BP for eIF4E. 4E-BPs share a common eIF4E binding motif with eIF4G, the scaffolding protein, making it a competitive inhibitor. Binding of 4E-BPs to eIF4E prevent interaction of eIF4G and eIF4E, thus impairing the assembly of the eIF4F complex. Hyperphosphorylated 4E-BPs have a high binding affinity for eIF4E and readily suppress translation. [35-36]

eIF4E and mTOR

mTOR is a 289-kDa serine/threonine protein kinase that phosphorylates 4E-BPs as well as other substrates when activated. mTOR controls many cellular processes including protein synthesis, cell growth, proliferation, mitochondrial function, and many other vital processes. [37] mTOR forms two separate complexes, mammalian target of rapamycin complex 1/2 (mTORC1/mTORC2). The two complexes share the following common subunits: LST8/GβL (lethal with sec18 protein 8/G-protein beta like subunit), DEPTOR (domain containing mTOR-interacting proteins), and Tti1/Tel2 complex. [38-40] Separate from mTORC2, mTORC1 contains the Raptor (regulatory-associated protein of mammalian target of rapamycin) subunit and PRAS40 (proline rich Akt substrate of 40 kDa). [41-42] mSin1 (mammalian stress-activated map kinase-interacting protein 1), Rictor (rapamycin-insensitive companion of mTOR), and Proctor (protein observed with Rictor, are all specific to mTORC2.

mTORC1 is activated by many extracellular signals including the presence of growth factors and stress as well as intracellular signals including changes energy status and oxygen levels. [22] mTORC1 is of particular interest as the Raptor domain serves as an adaption site for downstream substrates 4E-BPs. The PI3K/Akt/mTOR signaling cascade is responsible for phosphorylation of 4E-BPs via mTORC1. (Figure 5)

Phosphatidylinositol-3-kinase (PI3K) phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to form phosphatidylinositol-3,4,5-trisphosphate (PIP_3). [37] Membranous PIP_3 then activates protein kinase B (Akt) by binding to the PH domain which docks Akt to the plasma membrane where it is phosphorylated by many kinases- including mTORC2. [43] Active Akt phosphorylates TSC2, causing reduced activity, destabilization,

and dissociation from the TSC1/2 complex. TSC1/2 complex acts as a mTORC1 inhibitor. When the TSC1/2 complex is inactivated due to dissociation of TSC2, mTORC1 is activated. [44]

There are three known homologs of 4E-BP: 4E-BP1, 2, and 3. 4E-BP1 is the major isoform involved in regulation of nociception where 4E-BP2 is the dominant isoform in the brain. Active mTORC1 phosphorylates 4E-BPs. When hypophosphorylated, 4E-BPs compete with eIF4G for binding to eIF4E which represses eIF4F complex assembly and thus translation initiation. However, when phosphorylated by mTORC1, 4E-BPs dissociate from eIF4E allowing eIF4G to bind which completes the formation of the eIF4F complex allowing translation to proceed. [45] When 4E-BP1 is deleted in mice and eIF4E is free to interact with other eIF4F components, the mice show mechanical hypersensitivity, increased excitatory synaptic input, and a lowered threshold for synaptic potentiation. [46]

The mTORC1 cascade can be modulated by a multitude of internal and external cues. Two well studied signaling molecules that are involved in the activation of the mTORC1cascade are interleukin 6 (IL-6) and neurotrophic growth factor (NGF). IL-6 and NGF activate mTORC1 and, subsequently, promotion of 4E-BP1 phosphorylation. Intraplantar injection of IL-6 and NGF induced mechanical allodynia in mice. This phenomenon is mitigated through administration of mTORC1 inhibitor, rapamycin, as well as 4EGI-1. 4EGI-1 is a small molecule that is able to interrupt eIF4E/eIF4G interaction and thus formation of the eIF4F complex by competitively binding to eIF4E. 4EGI-1 also blocked hypersensitization after hyperalgesic priming with IL-6 and NGF. [24, 47, 48]

MNK Phosphorylation of eIF4E

It has been established that eIF4E plays an important role in the recognition of the 7-methylguanosine cap and formation of the eIF4F complex during cap-dependent translation of mRNA. eIF4E has a sole phosphorylation site at ser209 in mammals [49] and can exist in both phosphorylated and non-phosphorylated states. Phosphorylated eIF4E has a higher binding affinity to the 5' cap of untranslated mRNA. [50] Higher binding affinity of eIF4E to the 5' cap of mRNA results in increased assembly of the eIF4F complex and thus increased protein synthesis. [51]

Phosphorylation of eIF4E has also been shown to play a critical role in the development of cancer. Studies in mice have shown that phosphorylation of eIF4E is required for translational upregulation of several proteins implicated in tumorigenesis. Increased phosphorylation of eIF4E has also been associated with increased disease progression in prostate cancer. [52] eIF4E phosphorylation is not vital for development and survival but has been shown to be a critical component in various disease states and upregulation of the translation of a specific subset of mRNAs. Many mRNAs, including those involved in nociceptor plasticity and pain signaling are affected by phosphorylation of eIF4E. The significance of eIF4E phosphorylation in nociception, as mention earlier, was determined in a study that mutated the lone phosphorylation site, serine209, to alanine ($eIF4E^{S209A}$) to prevent the phosphorylation of eIF4E. These $eIF4E^{s209A}$ mutant mice showed reduced hypersensitivity and hyperalgesic priming. This result suggests that reducing the phosphorylation of eIF4E could provide an avenue in the production of pharmaceuticals aiming to treat cases of chronic nociceptor hypersensitization. [31] eIF4E kinases, such as

MNKs (MAPK interaction protein kinases) are an interesting target for the potential intervention of progressive diseases including chronic pain. [53, 54]

MNK 1 and 2 are serine/threonine kinases that can be activated by two different MAPK signaling pathways: ERK (extracellular regulated kinase) and p38 via various extracellular signals (Figure 5). MNK1/2 were originally discovered in a substrate screen for ERK1/2. [55] ERK1/2 are growth factor regulated kinases that interact downstream in the Ras/Raf/Mek/ERK phosphorylation cascade. When Raf is activated, it functions to phosphorylate MAPK/ERK kinases (Mek), which in turn phosphorylate ERK1/2. ERK1/2 then are able to activate and phosphorylate MNK1/2. [56] The p38 MAPK pathway is activated by stress signals including osmotic shock, UV radiation, cytokines, and chemokines. [53] When p38 is activated via phosphorylation it is able to activate and phosphorylate MNK1/2. [53]

Activated MNK1/2 acts a master translation regulator of eIF4E through direct phosphorylation on eIF4E's sole phosphorylation site, ser209. [49] Phosphorylation of eIF4E by its specific kinase, MNK, regulates translation of the eIF4E sensitive mRNAs. As established above, phosphorylated eIF4E plays a more active role in the eIF4F translation initiation complex. Many experiments have been done on mice that further investigated the role of eIF4E phosphorylation through MNKs on translation and the development of chronic pain. Mice lacking MNK1/2, and thus eIF4E phosphorylation, showed reduced development of mechanical and cold hypersensitivity following nerve injury. These results were validated through further experimentation which showed that local inhibition of MNKs was also able to reduce mechanical hypersensitivity as well as hyperalgesic priming in response to NGF. MNKs phosphorylation of eIF4E is necessary for phenotypic changes within neurons that are

necessary for the development of chronic pain. This further suggests that eIF4E sensitive mRNAs are likely targeted by eIF4E dependent changes in translation during the development of nociceptor hypersensitization. [22, 54, 57] eIF4E is a key potential therapeutic target in inhibiting abnormal translation initiation involved in many pathologies including cancers and chronic pain. eIF4E is a central node in regulating the rate at which translation occurs and its activity can be altered through both the MNK and mTOR signaling cascades in variety of different model organism.

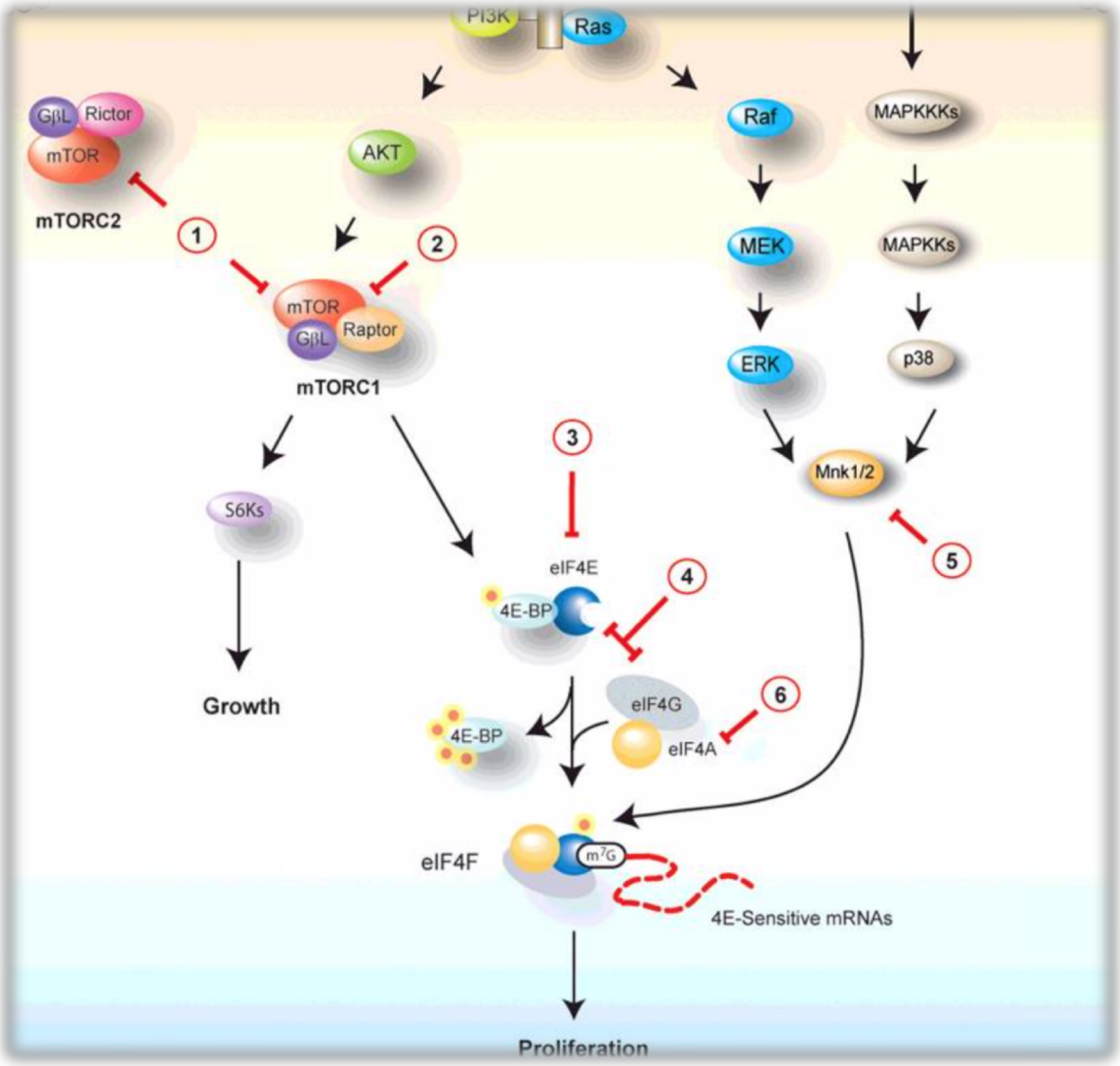

Figure 5. Multiple signaling pathways converge on eIF4E regulating translation initiation. Substrates mTOR and MNK are activated and phosphorylated by upstream signaling cascades which allows for regulation of eIF4E activity within the translation initiation complex eIF4F. [22]

Drosophila melanogaster as a Model Organism

To help illuminate the molecular mechanisms that underly the transition of nociceptors from normal nociception to states of chronic hypersensitivity, I am using the model organism *Drosophila* melanogaster. *Drosophila* has been used as a model organism for more than 100 years. [58]. The use of *Drosophil*a is ideal as it combines genetic, behavioral, and even economic advantages. *Drosophila* was one of the first organisms to have a fully sequenced genome that consist of approximately 13,600 protein-coding genes that are located on only four chromosomes. [59] It has been discovered that 75% of the coding genes are homologous to human disease-related genes. [60] *Drosophila* larvae are cheap to maintain when compared to other model organism and are very useful in biomedical research as they develop very quickly and have an average lifespan of ~120 day. The characteristic short lifespan allows for the effective study of gene expression on development nociception as the development from embryo to larvae can occur in as little as 4 days. [61] *Drosophila* is also useful in biomedical research due to the variety of well-established molecular genetics tools that have been established over its long history as an animal model. Advances in manipulation of the *Drosophila* genome allow for tissue-specific knockdown and fluorescent protein expression in specific cells without altering the surrounding tissue environment. [62]

Many researchers utilize *Drosophila* during the larval stage of development. The life cycle of *Drosophila* goes to completion within 10 days and consists of the following phases: embryo, larva, pupae, adult. [63] The larval phase can further be broken down into first, seconds, and third instar larval stages. The third instar and most mature larval stage is used for experiments as it has highly characterized behavior and is amenable to microscopy.

Under normal conditions third instar larvae will move forward in a peristaltic motion. However, upon sensing a threat, or noxious stimuli, the larvae with exhibit nocifensive escape locomotion (NEL), which consist of a 360º barrel role along the longitudinal axis toward or away from a noxious stimulus. NEL behavior is an evolutionarily conserved mechanism that protect larva from predation, most commonly, predation from parasitoid wasps. This NEL behavior is quantifiable, which allows larval sensitivity to noxious stimuli to be measured. [15] It is also ideal that third instar larvae have a transparent epidermis that allows for easy imaging and analysis of florescent expressing cells, such as green fluorescent protein (GFP) expressing neurons, within the epidermis to study their fully developed nervous system. [64]

The overarching pathways that underly the transmission of signals from nociceptors is conserved from *Drosophila* to humans. In both humans and flies, action potentials from the PNS are triggered by noxious stimuli and transmitted from activated nociceptors, through the axon, and to the CNS. Upon reaching the CNS, nociceptors synapse with secondary neurons and transmit the signal to higher-order processing centers. [65]

The *Drosophila* sensory nervous system receives and transduces information from the outside environment to the CNS in order to promote appropriate larval and adult behavior. Somatosensory system inputs are diverse, and can be reflected by the many different morphologies of individual sensory organs. Sensory organs can be categorized most loosely in dorsal(d), lateral(l), ventral(v), and ventral'(v') clusters. The clusters consist of neurons that are classified as either type I, sensilla, or type II, multidendritic (md) neurons. Type I neurons include the mechanosensory external sensory organs. Type II, md neurons spread complex, highly branched dendritic processes and are characterized by free nerve endings

and that innervate external surfaces, such as the epidermis, and some internal surfaces. [66] Md neurons can further be classified by neurons that give rise to elaborate dendritic arborizations (da neurons), neurons that have bipolar dendrites (bd neurons), and neurons that arborize around particular tracheal branches (td neurons). The arrangement of the segmented peripheral neurons is highly invariant and provides a favorable system for the genetic analysis of neurodevelopment. [67]

Among the mdda (multidendritic-dendritic arborization) neurons, which extend dendrites across the epidermis, there is considerable morphological diversity. Four distinct morphological classes of mdda neurons have been distinguished in *Drosophila* larva, primarily by their physiological function and patterns of dendritic complexity. The four classes are labeled as class I, II, III and IV neurons. A higher class-number is indicative of increasing arbor complexity. Class I and class II neurons have simple branching patterns, class III show numerous short actin-based protrusions extending from major branches, and class IV neurons innervate the entire epidermis with complex, space filling arbors. [68] (Figure 6) Molecular and functional properties correlate with these morphological distinctions. Class I neurons function as proprioceptors responding to stimuli from within the body, coordinating body position and movement. [69-70] Class II and III neurons function as touch receptors that respond to gentle touch. [71] Class IV neurons function as polymodal nociceptive (noxious sensing) neurons.[72]

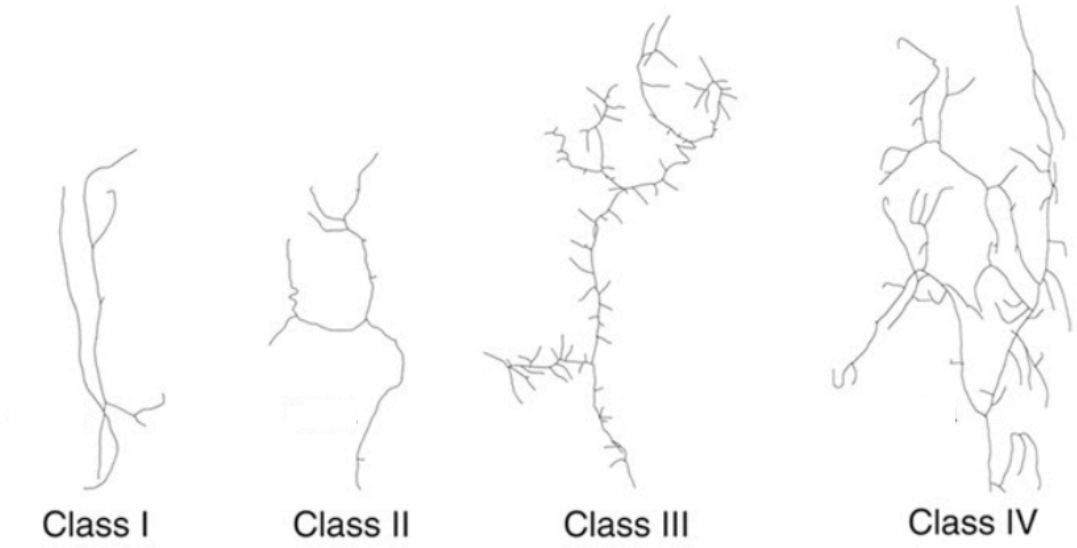

Figure 6. Classes of multidendritic dendritic arborization neurons in *Drosophila.* Class I and class II neurons have simple branching patterns, class III show numerous short actin-based protrusions extending from major branches, and class IV neurons innervate the entire epidermis with complex, space filling arbors. [68]

Ion Channels Allow for Signal Transduction in Drosophila

Dendrites of Mdda class IV neurons are the primary site for noxious sensory input in *Drosophila.* These dendrites transduce sensory input through a nociceptive pathway similar to the one in vertebrates described previously. (Figure 1). For most secondary neurons that receive afferent information, proper function of the pathways generating and propagating action potential within dendrites is essential. [63]

Transient receptor potential ion channels (TRP channels), such as TRPA1, are expressed by nociceptive neurons are involved in peripheral sensitization and the development of chronic pain. [73] TRP channels were originally discovered and identified as important factors in sensory transduction in studies using *Drosophila melanogaster*. TRP channels are a family of ion channels that act as sensors of the environment and integrate signaling, and their expression in neurons determines the activation of nociceptors by different noxious stimuli. This group of channels utilize an ion flux as a result of activation by noxious stimuli. This causes neuronal membrane depolarization, which activates action potentials. [74-75]

Specific to *Drosophila,* activation of TRPA channels in class IV mdda neurons results in the larval NEL response. [76] In order to understand more about the role of the nociceptive TRPA channels, many studies focused on the function of TRPA1. To better understand the role of TRPA1 in *Drosophila* nociception, an RNAi knockdown of *TrpA1* was conducted. It was found that the knockdown larvae exhibited impaired avoidance of noxious heat, showing it is required for thermal nociception. [74] TRPA1 has also been studied in mice in which it was found that TRPV1 functions in the transmission of thermal nociceptive signal in response to noxious heat in the range of 42^{o}C-48^{o}C. The TRPA1 channel is essential for

proper thermal nociception and is a characteristic that is conserved from to *Drosophila* to animal models. [15, 76, 77].

Responsible for the generation and conduction of electrical signals in neurons, voltage-gated sodium (Na_v channels) are essential for neural signaling. When Na_v channels are pharmacologically blocked, all receptor and ion channel-induced currents are halted at the axon and further prevented from signaling to the secondary neuron [78]. In *Drosophila* neurons, the sodium voltage-gated ion channel, *paralytic* (*para*), is required for nociceptor activity. When *para* is depleted, nociceptors cannot fire action potentials and the NEL response to noxious stimuli is completely lost [79-80]. Knockdown of *Para* in nociceptors is often used a positive control for reduced sensitivity of nociceptors in studies using *Drosophila.*

Tools for studying the complexity of nociception in *Drosophila* have advanced in recent years in hopes to establish further clarity in conserved processes of nociception. A model of UV-induced hypersensitization has been developed in *Drosophila* larvae that has proven useful in research. This UV-induced sensitization model can be used to assess whether the cellular and molecular mechanisms underlying nociceptive sensitization differ from those that underlie baseline nociception. In this model, *Drosophila* larvae are exposed to UV radiation which causes epidermal tissue-damage resulting in increased sensitivity of nociceptors. UV-induced sensitization has also been effective in establishing the role of gene expression on various aspects of hypersensitization such as allodynia and hyperalgesia. A recent study was able to show that reduced function of the *Drosophila* homolog of TNF-α, an evolutionarily conserved molecule that is released from damaged epidermal cells whose receptor is found within nociceptors, was able to mitigate the development of thermal

allodynia while having no effect on thermal hyperalgesia following UV-sensitization. This model can further be used to classify the role of other genes, such as *Lk6* and *Tor*, on nociception in *Drosophila.*

With a highly characterized nervous system, class IV mdda neurons as nociceptors that transmit sensory information through various ion channels, well-developed tools of genetic manipulation, and established methods for testing changes in nociception, *Drosophila* becomes an ideal organism for the study of nociceptive plasticity through translational control. This research utilized the insertion of a transgene in combination with a tissue specific driver to cell-specifically knockdown two genes of interest. The genes of interest are *Lk6* and *Tor*, the *Drosophila* homologs of previously- mentioned kinases, *mTOR* and *MNK,* respectively. With this information, is was possible to study how changes in translation regulation and development though *Lk6* and *Tor* loss of function effected nociception. [81-82]

Specific Aims:

I. Define baseline thermal and mechanical larval sensitivity when target genes, *Lk6* or *Tor* is knocked down.
II. Quantify the effects of *Lk6* and *Tor* knockdown on nociceptor morphology.
III. Characterize the effect of *Lk6* knockdown on sensitization.

Materials and Methods

Drosophila Genetics

The goal of the project was to examine the role of specific kinases in class IV mdda nociceptors in development and function. In order to examine the role of the kinases, RNA interference (RNAi) knockdown lines for each gene were crossed with flies containing tissue specific drivers to knockdown (KD) the expression of the genes specifically in class IV mdda neurons.

RNAi is a gene silencing method that works through degradation of homologous messenger RNAs (mRNA). RNAi is an endogenous cellular mechanism triggered by double stranded RNA (dsRNA), which leads to the degradation of homologous RNAs. [83] dsRNA is produced through transcription of an inverted repeat or "hairpin" sequence. Upon introduction of dsRNA, the formation of a complex, consisting of Dicer-2 and R2D2, cuts the duplex RNAs into short fragments are 19-21 nucleotides. [84] This in turn induces the association of the argonaute protein, Ago2, which is stabilized by a HSC70/Hsp90 chaperone system, and then leads to the unwinding of the duplex, its cleavage, and finally ejection of the passenger strand [85] Subsequently, the full RNA-induced silencer complex (RISC) is formed. This complex identifies sequence-homologous endogenous RNAs through a homology-seeking activity, leading to their cleavage and degradation.

Cell-specific RNAi was used to knock down transcript levels in the nociceptor by utilizing the binary GAL4/upstream activation sequence (UAS) system. The GAL4/UAS system is a genetic tool used to control cell-specific gene expression levels in *Drosophila*. To generate transgenic lines expressing GAL4 in various cell and tissue-specific patterns, the GAL4 gene is inserted randomly into the genome, driving GAL4 expression from various

different genomic enhancers. A GAL4-dependent target gene, in this case a UAS-RNAi transgene, can then be constructed by subcloning any sequence behind a GAL4 binding site. The target gene is silent in the absence of GAL4. To activate the target gene in the cell or tissue specific pattern, flies carrying the target (UAS-Gene X) are crossed to flies expressing GAL4 in the tissue of interest. In the progeny of this cross, it is possible to activate UAS-Gene X in cells where GAL4 is expressed and to observe the effect of this directed misexpression in the case of RNAi knockdown, or on development. [2]

The GAL4 driver used in this experiment is the *pickpocket1.9*-GAL4 (*ppk*-GAL4) driver which targets expression to class IV mdda neurons. *Pickpocket1* (PPK1) is a *Drosophila* subunit of the epithelial sodium channel (ENaC) family displaying limited expression in mdda neurons. [87] The advantage of cell or tissue specific gene knockdown is that the cell is not completely deprived of the targeted protein; the system allows for expression in non-targeted cells and an increased chance of cell viability. To decrease gene expression in nociceptors, *ppk1.9-GAL4;UAS-dicer-2* flies were crossed to *UAS*- RNAi lines for each gene of interest. (Figure 7) The RNAi degrades mRNA transcripts to result in reduced levels of the target protein while the presence of *dicer-2* increases RNAi transcript activity. However, the efficiency of cell or tissue specific gene knockdown was not confirmed in this project, so the possibility of false positive or negative phenotypes due to off-target effects or leaky expression was present. [88]

The presented research used crosses that consisted of *ppk-GAL4;UAS-dicer-2* driver combined with *UAS-RNAi* to knockdown the genes of interest. The driver stocks *w; ppk1.9-GAL4; UAS-dicer2*, *w; ppk1.9-GAL4, UAS-mCD8::GFP; UAS-dicer2*, and the w^{1118} control were obtained as gifts from the laboratory of the Dr. Dan Tracey. All other RNAi lines were

developed as part of the *Drosophila* Transgenic RNAi Project (TRiP) and obtained from the Bloomington *Drosophila* Stock Center (BDSC). (Table 1)

Two negative controls were used to monitor the fidelity of the GAL4/UAS system, controlling for each portion of the binary system. The first negative control controlled for the GAL4 insertions. Virgin females from the GAL4 (*w; ppk1.9-GAL4; UAS-dicer2*) driver line were crossed with males from the control (y^1 v^1*; P{y[+t7.7]=CaryP}attP2* (BDSC# 36303)) line. The second negative control controlled for the insertion of the following UAS-RNAi transgenes: *Tor*-RNAi, y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMS00904}*attP2* (BDSC# 33951); *Tor*-RNAi, y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$= TRiP.GL00156}*attP2* (BDSC# 35578); *Tor*-RNAi, y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$= TRiP.HMS01114}*attP2* (BDSC# 34639); *Lk6*-RNAi, y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$= TRiP.GL00264}*attP2* (BDSC# 35352); *Lk6*-RNAi, y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$= TRiP.JF02993}*attP2/TM3, Sb*^{1} (BDSC# 28357). Virgin females from the control line (w^{1118}) were crossed with males from the previous listed RNAi lines.

A single positive control was generated by crossing Virgin females from the GAL4 (*w; ppk1.9-GAL4; UAS-dicer2*) driver line with males from the *para*-RNAi line. *Para* knockdown larvae were used as a positive control and found to show nearly complete insensitivity to noxious thermal stimuli. *Paralytic (para),* is a *Drosophila* ion channel gene that encodes for components of voltage-gated sodium channels that are required for action-potential propagation in embryonic and larval motor neurons. [89]

Drosophila Husbandry

All larvae were reared on cornmeal-molasses medium (Nutri-Fly M; Genesee Scientific, El Cajon, CA, USA). Each cross was reared using 6 virgin females from a driver

line and 3 males from either an RNAi or control line. Flies and larvae in all experiments were grown in a 25 °C incubator that was maintained at ~50% humidity and set to a timed 12-hour light/12-hour dark cycle. Larvae for all experiments were obtained by washing wandering 3rd instar larvae from the walls of vials using distilled water. Once washed from the vials, the larvae were allowed to acclimate for 3-5 minutes in a thin film of distilled water and yeast. Further preparation for assays is described in detail below.

Thermal Nociception Assay

Thermal nociception assays were conducted as previously described. [21] Wandering 3rd instar larvae were washed from vials into glass petri dishes using distilled water. A small amount of dry baker's yeast (3-5 mg) and ~2ml of distilled water was added to each petri dish. The yeast served to disrupt surface tension of the water. Once the yeast had dissolved into the water, enough water was removed that only a thin film remained. Larvae were then stimulated along their lateral surface with a custom-built thermal probe consisting of a soldering iron with a tip filed into a ~6 mm chisel shape. The custom probe was plugged into a Variac Variable Transformer (Part No. ST3PN1210B) (ISE, Inc., Cleveland, OH) to control the temperature of the probe. An IT-23 thermistor and BAT-12 digital thermometer (Physitemp, Clifton, NJ) was used to digitally monitor probe temperature in real time. The thermistor and thermometer provided temperature information to a tenth of a degree. During experiments, the probe tip temperature was monitored before and after application of the stimulus. Trials in which the probe temperature deviated from the desired temperature by ±1 °C were discarded. Baseline thermal nociception assays were conducted at 46 ± 1 °C while injury-induced hypersensitization experiments tested larval thermal nociception at

42 ± 1 °C. Behavioral responses were recorded at 30 frames per second using a digital video camera mounted on a dissecting microscope. Adobe Premier Pro was used to determine the latency. The latency was considered to be the difference in the time it took for the larvae to complete NEL from the time the probe made contact with the lateral surface. Larvae that did not respond to the thermal stimulus within eleven seconds were scored as 11 for all subsequent analysis. 50 larvae were tested for each genotype in each experiment. Each cross was blinded to larval genotype during testing and analysis. The non-parametric Mann-Whitney U test was used to determine statistical significance of latency differences between control and experimental genotypes. Bonferroni correction was used to correct the α value for multiple comparisons in experiments with more than one experimental group compared to the control. With one experimental group, an *α value of 0.05 was used, while with two experimental groups, an α value of 0.025 was used.*

Mechanical Nociception Assay

Mechanical nociception assays were conducted as previously described. [90] Larvae for the mechanical assays were prepped in the same fashion as the thermal assay. Mechanical stimulation was applied using a custom-built Von Frey filament made from 8 lb. test nylon fishing line (Stren Original Monofilament 8 lb. line, Part #1304152, Pure Fishing, Inc., Columbia, SC) measured and cut to 10 mm and taped to a Pasteur pipette. The Von Frey filament delivers a force of ~50 millinewtons (mN). Each larva was stimulated with the same custom Von Frey filament. The NEL response was then recorded using a binary, response or no response system in order to calculate the percentage of larvae that responded. Each larva was stimulated three times. The first stimulus was the only stimulus used for scoring and

subsequent analyses. 100 larvae were tested per genotype in each experiment. Experimenters were blinded to larval genotype during stimulation. The Chi-square test was used to test for statistically significant differences in proportion of responders between control and experimental genotypes. Bonferroni correction was used to correct the α value for multiple comparisons in experiments with more than one experimental group compared to the control. With one experimental group*, an* α value of 0.05 was used, while with to experimental groups, an α value of 0.025 was used.

Confocal Microscopy Assay

Wandering 3rd instar larvae obtained from the walls of the vile using a fine tip paint brush and rinsed using distilled water in a petri dish. Using dissection microscope and forceps, the larvae were immobilized by circumferential ligation using a human hair tied tightly around segment A3. This method paralyzed all body segments posterior to the ligation. Immobilized larvae were then mounted in glycerol between two glass coverslips and image using confocal microscopy. A Zeiss LSM 880 microscope with a 488 nm laser line was used for all images. Tiled z-stacks were obtained in order to capture the full arborization of class IV mdda neurons from body segments posterior to the ligation. Dendrites were analyzed via Sholl analysis using the NeuronJ plugin for ImageJ. [91] To use this software, background noise from images was removed and dendrites were manually traced using Adobe Photoshop. Sholl Analysis creates a series of concentric circles and automatically quantified the average number of dendritic intersections per concentric circle as well as the total sum of all dendritic intersection with the concentric circles. 5-10 larvae were tested for each genotype in each experiment. A paired *t*-test was used to test for statistically significant

differences between control and experimental conditions. An α value of 0.05 was used to determine statistical significance.

Hypersensitization Assay

Wandering 3rd instar larvae were washed from the walls of the vile using distilled water and discarded to reduce the number of pupation events during the recovery. Using distilled water, early 3rd instar larva and late 2nd instar larvae were washed from the food into a petri dish. All food and debris were removed from the larva using distilled water. The larvae from each genotype were then divided into separate groups (UV-exposure and NO UV-exposure) on separate petri dished. All petri dishes were places on ice for ~30 seconds to anesthetize the larvae to eliminate movement and overlap of larva during UV-exposure. The UV-exposure groups were placed into the UV Crosslinker and subjected to UV exposure of 25 MJ/cm2 at 254 nm. The NO UV-exposure groups were placed into the UV Crosslinker and subjected to no UV. The larvae were then transferred using a fine tip paint brush to room temperature apple cider vinegar agar plates that had a thin film of yeast paste for recovery. The yeast paste was made by combining 2 parts dry Baker's yeast with 1part distilled water. The recovery period occurred in the 25 °C incubator that was maintained at ∼50% humidity. Following an 8-hour recovery period, the larvae were washed with distilled water from the apple cider vinegar agar plates into a petri dish. A thermal nociception assay was then performed. 50 larvae were tested for each genotype under both UV and NO UV conditions. Larvae were tested for hypersensitization using the thermal nociception assay analysis method. A non-parametric Mann-Whitney U test was used to determine statistical significance with an α value of 0.05.

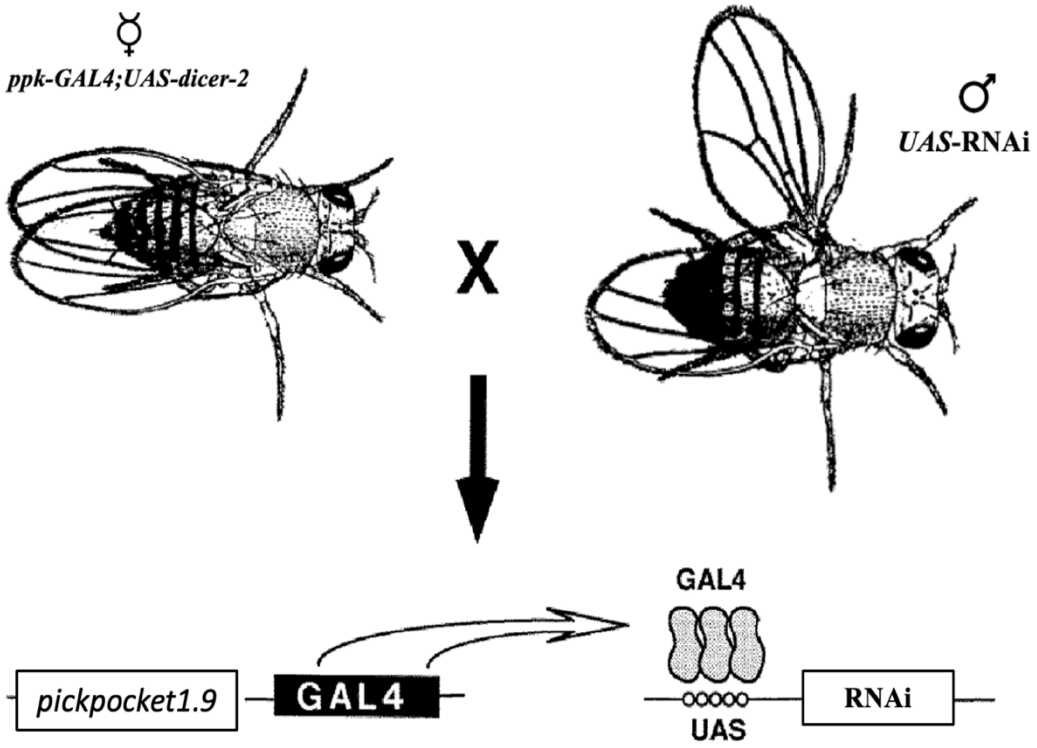

Figure 7. Adapted from Brand et al. 1993. [86] Transcriptional activation of RNAi via tissue specific expression of GAL4 using Class IV neuron cell specific enhancer, *pickpocket1.9*.

Table 1. *Drosophila melanogaster* stocks used for thermal, mechanical, hypersensitization, and confocal microscopy assays. (BDSC: Bloomington *Drosophila* Stock Center) (VDRC: Vienna *Drosophila* Resource Center)

Gene	Origin	Genotype	Notes
ppk-GAL4; UAS-dicer-2	Dan Tracey Lab	w^{1118}; *ppk*-GAL4; UAS-*dicer2*	GAL4 Driver
UAS-mCD8::GFP; UAS-dicer2	Dan Tracey Lab	*UAS-mCD8::GFP; UAS-dicer2*	GFP Driver
Control for TRiP RNAi	BDSC# 36303	y^1 v^1; *P{y*$^{+t7.7}$*=CaryP}attP2*	Negative Control
w^{1118}	Dan Tracey Lab	w^{1118}	Negative Control
UAS-para-RNAi	Dan Tracey Lab (VDRC# 6139)	W^{1118}; P{*UAS-para*-RNAi}	Positive Control
Tor-RNAi	BDSC# 33951	y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$= TRiP.HMS00904}*attP2*	
Tor-RNAi	BDSC# 35578	y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$= TRiP.GL00156}*attP2*	
Tor-RNAi	BDSC# 34639	y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$= TRiP.HMS01114}*attP2*	
Lk6-RNAi	BDSC# 35352	y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$= TRiP.GL00264}*attP2*	
Lk6-RNAi	BDSC# 28357	y^1 sc^* v^1 sev^{21} P{$y^{+t7.7}$ $v^{+t1.8}$= TRiP.JF02993}*attP2/TM3,* Sb^1	

Results

To better understand the role of eIF4F-regulating kinases *Lk6* and *Tor* on post-transcriptional regulation of proteins involved in nociception, the genes encoding *Lk6* and *Tor* were knocked down in class IV mdda neurons through UAS-RNAi driven using the nociceptor-specific driver line *ppk1.9*-GAL4*;* UA*S-dicer-2.* For all experiments, two negative controls were used to identify the effect of each portion of the binary GAL4/UAS system on thermal or mechanical nociception. Each negative control tested one element of the binary GAL4/UAS system. The GAL4-only control, labeled ppk/+ in figures, expressed the *ppk1.9*-GAL4; UAS-*dicer-2* and no UAS-RNAi. The UAS-only control, labeled UAS-"*Gene*"-RNAi/+ in figures, contained the UAS-RNAi portion of the binary system with no *ppk1.*9-GAL4; UAS-*dicer-2*. For all thermal experiments, an increased average latency indicated hyposensitivity or decreased sensitivity to a noxious thermal stimulus, where a decreased average latency indicated hypersensitivity or increased sensitivity to a noxious thermal stimulus. For all mechanical experiments, an increase in the number of individuals to respond (% response) to a noxious mechanical stimulus indicated hypersensitivity, where a decrease in the number of individuals to respond (% response) to a noxious mechanical stimulus indicated hyposensitivity. For hypersensitization experiments, decreased average latency in response to a noxious mechanical stimulus indicated injury-induced hypersensitization via UV radiation.

Lk6 knockdown does not have an effect on baseline nociceptor sensitivity

To examine the role of *Lk6* in baseline thermal nociceptor sensitivity, *Lk6* was knocked down in *Drosophila* class IV mmda neurons using the GAL4/UAS system

previously described. *Lk6* knockdown larvae using RNAi transgene BDSC# 35352 had an average latency of 2.79 seconds (s); *Lk6* knockdown larvae using RNAi transgene BDSC# 28357 had an average latency of 3.02 s, while the GAL4 only, UAS-*Lk6*-RNAi/+ (BDSC# 35352), and UAS-*Lk6*-RNAi/+ (BDSC# 35352) had average latencies of 2.94 s, 3.28 s, and 3.36 s respectively. Using a non-parametric Mann-Whitney U statistical test with an α value of 0.025, it was shown that *Lk6* knockdown larvae using either RNAi transgene BDSC# 35352 or RNAi transgene BDSC# 28357 had no significant difference in latencies when compared to both negative controls, the GAL4 only control ($p=0.385$ and $p=0.847$) and the respective UAS-*Lk6*-RNAi controls ($p=0.207$ and $p=0.523$). Both experimental groups and all three negative controls have a significantly lower latency than the positive, UAS-*para*-RNAi control which had an average latency of 11 s. Larvae with nociceptor-specific knockdown of *Lk6* showed no changes in their ability to detect a noxious thermal stimulus (46°C). (Figure 8)

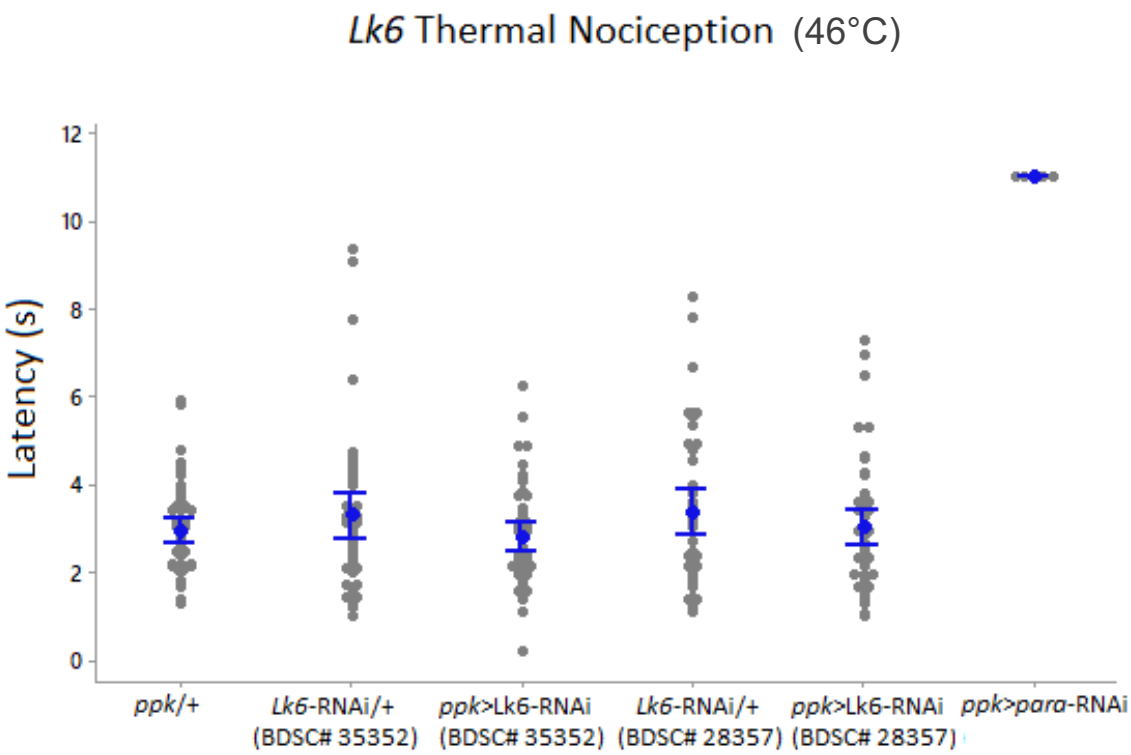

Figure 8. Larvae with nociceptor-specific knockdown of *Lk6* showed no significant difference in their response latency to a noxious thermal stimulus (46°C) when compared to GAL4 only controls (ppk/+) as well as UAS-RNAi only negative controls. UAS-RNAi only negative controls also showed no significant difference in their response latency to a noxious thermal stimulus when compared to the GAL4 only negative control. Larvae with nociceptor-specific knockdown of *para* showed severely impaired nociceptive responses (increase latency) and were used as a positive control. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as an enlarged point with an interval bar representing the standard error. (n = 50 for all groups; significance determined by non-parametric Mann-Whitney U test; α value of 0.025 was used).

To examine the role of *Lk6* in baseline mechanical nociceptor sensitivity, *Lk6* was knocked down in *Drosophila* class IV mdda neurons using the GAL4/UAS system previously described. *Lk6* knockdown larvae using RNAi transgene BDSC# 35352 resulted in 40% of the larvae responding to the noxious mechanical stimuli. *Lk6* knockdown larvae using RNAi transgene BDSC# 28357 resulted in 39% of the larvae responding to the noxious mechanical stimuli, and the GAL4 only control resulted in 51% of the larvae responding to the noxious mechanical stimuli. Using a Chi-square statistical test with an α value of 0.025, decided using the Bonferroni correction for multiple comparisons, it was shown that *Lk6* knockdown larvae using either RNAi transgene BDSC# 35352 or RNAi transgene BDSC# 28357 had no significant difference in % response when compared to the GAL4 only control ($p = 0.118$ and $p = 0.088$). Larvae with nociceptor-specific knockdown of *Lk6* showed no changes in their ability to detect a noxious mechanical stimulus (50 mN) when compared to the GAL4 only negative control. (Figure 9)

However, when UAS-*Lk6*-RNAi only controls were compared with both their respective experimental *Lk6* knockdown group and the GAL4 only control in the baseline mechanical assay, a significant decrease in % response was seen. UAS-*Lk6*-RNAi/+ (BDSC# 35352) resulted in 19% of the larvae responding to the noxious mechanical stimuli, and had a significantly decreased response when compared to *Lk6* knockdown larvae using RNAi transgene BDSC# 35352 ($p = 0.001$) and the GAL4 only control ($p = 0.000$). Similarly, UAS-*Lk6*-RNAi/+ (BDSC# 28357) resulted in 20% of the larvae responding to the noxious mechanical stimuli, and had a significantly decreased response when compared to *Lk6* knockdown larvae using RNAi transgene BDSC# 28357 (p $p = 0.003$) and the GAL4 only control ($p = 0.000$). This result was unexpected. If the GAL4/UAS system for cell-specific

knockdown behaving as expected, the UAS-*Lk6*-RNAi only controls would not be significantly different than the GAL4 only negative controls. Further experimentation must be done to determine if *Lk6* plays a role in class IV mdda neurons for baseline mechanical sensitivity. (Figure 9)

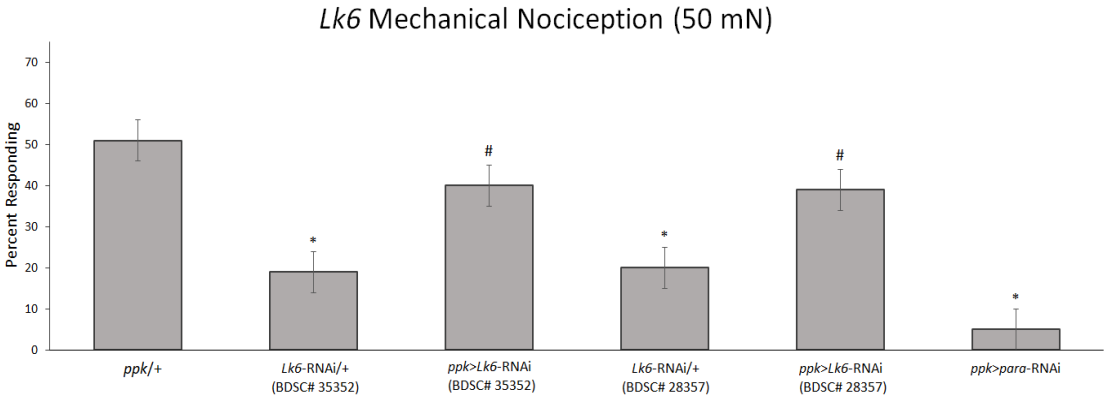

Figure 9. Larvae with nociceptor-specific knockdown of *Lk6* show no significant behavioral defect when compared to GAL4 only (ppk/+) negative controls. UAS-RNAi only controls showed significantly impaired baseline behavioral response to mechanical stimuli when compared to the GAL4 only negative control. UAS-RNAi only negative control larvae also showed a very low response rate when compared to their respective nociceptor-specific *Lk6* knockdown larvae. Larvae with nociceptor-specific knockdown of *para* also showed a very low rate of nociceptive responses and were used as a positive control. (n = 100 for all groups; * indicates a significant difference when compared to the GAL4 only negative control; # indicates a significant difference when compared to respective UAS-RNAi only negative controls. $p < 0.025$ by Chi-Square Test)

Lk6 knockdown mitigates effects of UV injury-induced hypersensitization

Previous studies in mice have shown that though *MNK* is not involved in baseline nociceptor sensitivity, it may play a role in the development of hypersensitization following tissue damage. [31] In order to determine if *Lk6* plays a role in nociceptor hypersensitization following tissue damage in the *Drosophila* model, a UV injury hypersensitization assay was used. [81] In this experiment, sensitivity of 3rd instar larvae to a noxious thermal stimulus (42°C) was tested following UV-exposure (25 MJ/cm2) and an 8-hour recovery period. The latency of NEL response to the thermal stimulus was quantified and analyzed as stated in the thermal nociception assay method. The data was categorized into percentage groups representing a slow, intermediate, and fast response (>10 s, 5-10 s, and <5 s) for purposes of visual interpretation. (Figure 10)

For each genotype there was a control, "NO UV", condition and an experimental, "UV" condition. Like previous experiments, each nociceptor-specific knockdown of *Lk6* (*ppk*>Lk6-RNAi) group has two negative controls, a GAL4 only (*ppk*/+) and a UAS-*Lk6*-RNAi only (UAS-*Lk6*-RNAi/+) group. Unlike previously experiments, statistical comparisons were only made between differing conditions of the same genotype. Development of hyperalgesia was determined through statistical analysis of the "UV" larvae versus the "NO UV" larvae of the same genotype. In the GAL4 only group, the average latency to respond to a noxious thermal stimulus was significantly decreased post UV exposure ($p = 0.044$). The GAL4 only group had an average response latency of 8.21 s with "NO UV" and 7.07 s with "UV". This decrease in average response latency, or increase in sensitivity, indicated that tissue damage induced hypersensitization did occur. The UAS-*Lk6*-RNAi only control using RNAi transgene BDSC #28357 also showed an increase in

sensitivity (p=0.018) UAS-*Lk6*-RNAi/+ (BDSC# 28357) larvae with "NO UV" had an average response latency of 8.49 s, while UAS-*Lk6*-RNAi/+ (BDSC# 28357) larvae with "UV" had an average response of 6.91 s. The UAS-*Lk6*-RNAi only control using RNAi transgene BDSC #35352 showed a trend toward increased sensitivity however there was no statistical evidence ($p = 0.193$). UAS-*Lk6*-RNAi/+ (BDSC# 35352) larvae with "NO UV" had an average response latency of 7.95 s, while the UAS-*Lk6*-RNAi/+ (BDSC# 35352) larvae with "UV" had an average response of 6.73 s.

Both groups with nociceptor-specific knock of *Lk6* showed no significant difference in sensitivity to a noxious thermal stimulus after UV induced tissue damage (BDSC# 28357 p= 0.764; BDSC#35352 $p = 0.444$). *Lk6* knockdown larvae with "NO UV" using RNAi transgene BDSC# 28357 had an average response latency of 7.88 s, while *Lk6* knockdown larvae with "UV" using RNAi transgene BDSC# 28357 had an average response of 7.78 s. *Lk6* knockdown larvae with "NO UV" using RNAi transgene BDSC# 35352 had an average response latency of 5.44 s, while *Lk6* knockdown larvae with "UV" using RNAi transgene BDSC# 28357 had an average response of 6.01 s. This data is indicative of nociceptor-specific knockdown of *Lk6* being associated with mitigated development of injury-induced hypersensitization.

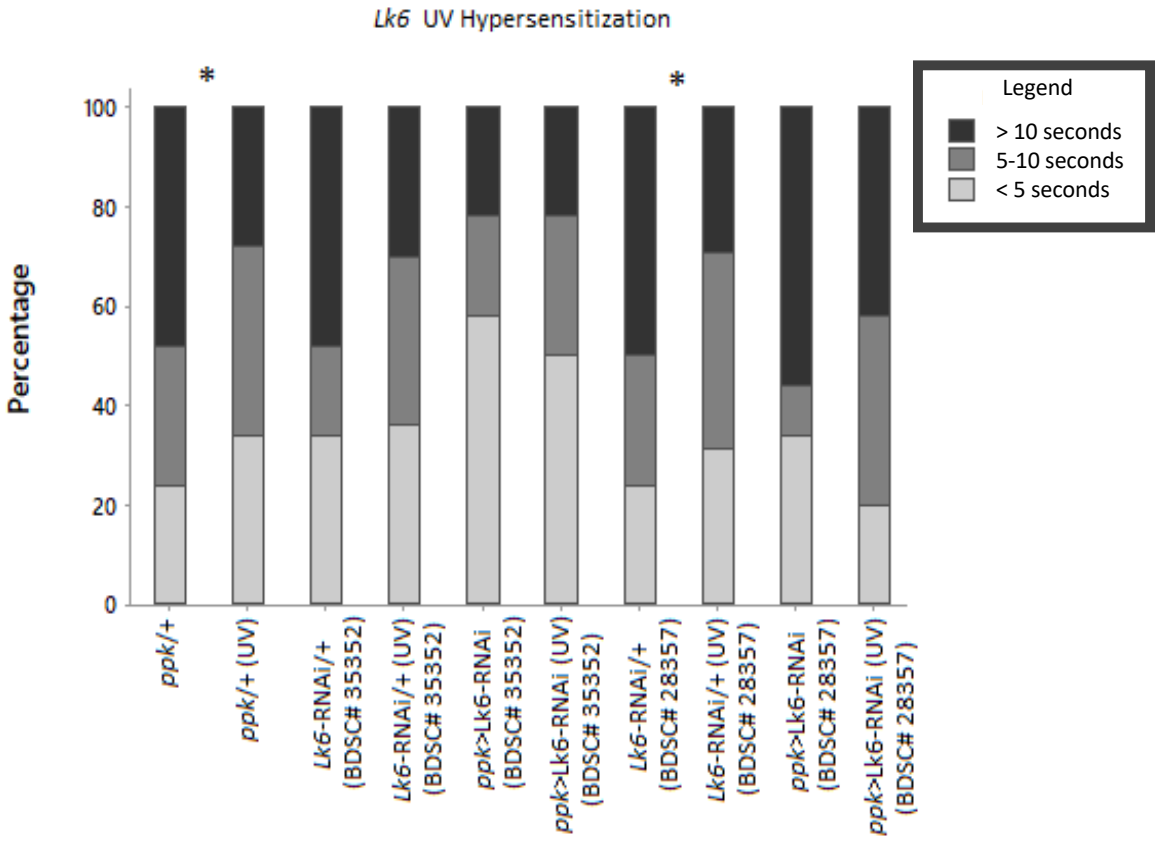

Figure 10. Nociceptor sensitization is decreased when in larvae with nociceptor-specific knockdown of *Lk6* post UV injury. Categorical NEL responses are represented as a percent of the total sample size (>10 seconds for slow response; 5-10 seconds for intermediate response; <5 for fast response). Larvae from the GAL 4 only (ppk/+) control group became more sensitive to a noxious thermal stimulus (42°C) and had a significantly reduced response latency post UV exposure. Larvae from the *Lk6*-UAS-RNAi (BDSC# 35352) only control for did not become sensitive to a noxious thermal stimulus (42°C) post UV exposure and showed no significant difference in latency when compared to the larvae without UV exposure. Larvae with nociceptor-specific knockdown of *Lk6* (BDSC# 35352) showed no significant difference in their response latency to a noxious thermal stimulus (42°C) post UV exposure when compared to larvae with nociceptor-specific knockdown of *Lk6* (BDSC# 35352) without UV exposure. Larvae from the *Lk6*-UAS-RNAi (BDSC# 28357) only control became more sensitive to a noxious thermal stimulus (42°C) and had a significantly reduced response latency post UV exposure when compared to *Lk6*-UAS-RNAi (BDSC# 28357) larvae without UV exposure. Larvae with nociceptor-specific knockdown of *Lk6* (BDSC# 28357) showed no significant difference in their response latency to a noxious thermal stimulus (42°C) post UV exposure when compared to larvae with nociceptor-specific knockdown of *Lk6* (BDSC# 28357) without UV exposure

Lk6 knockdown has no effect on nociceptor development

Defects in nociception and hypersensitization can potentially arise from defects in morphological development of class IV mdda neurons. Therefore, it was important to identify whether nociceptor-specific knockdown of *Lk6* was associated with any defects in dendritic complexity. To determine if defects in nociceptor plasticity in *Lk6* knockdown larvae stemmed from developmental defects, the expression of *GFP* and *Lk6* knockdown in class IV neurons was driven by *ppk1.9-GAL4>mCD8::GFP;*UAS-*dicer 2*. Live cell confocal microscopy images (Figure 11A/B) were prepared in Adobe Photoshop and analyzed using Sholl analysis. Sholl analysis provides quantitative information regarding the complexity of dendrites through methods involving the measurement of interaction of dendrites with a series of concentric circles. (Figure 11C/D)

Control (y^1 v^1; *P{*$y^{+t7.7}$*=CaryP}attP2*) larvae expressing GFP had an average of 26,185.5 total dendritic interactions while *Lk6* knockdown larva (BDSC# 35352) has an average of 29,515.9 total dendritic interactions (p=0.553). (Figure 11E) Control (y^1 v^1; *P{*$y^{+t7.7}$*=CaryP}attP2*) larvae expressing GFP had an average of 22.4 mean dendritic interactions while *Lk6* knockdown larva (BDSC# 35352) has an average of 26.7 mean dendritic interactions ($p = 0.112$). Significance determined by a paired *t*-test revealed that nociceptor-specific knockdown of *Lk6* was not associated with any development defects of class IV neurons. (Figure 11F)

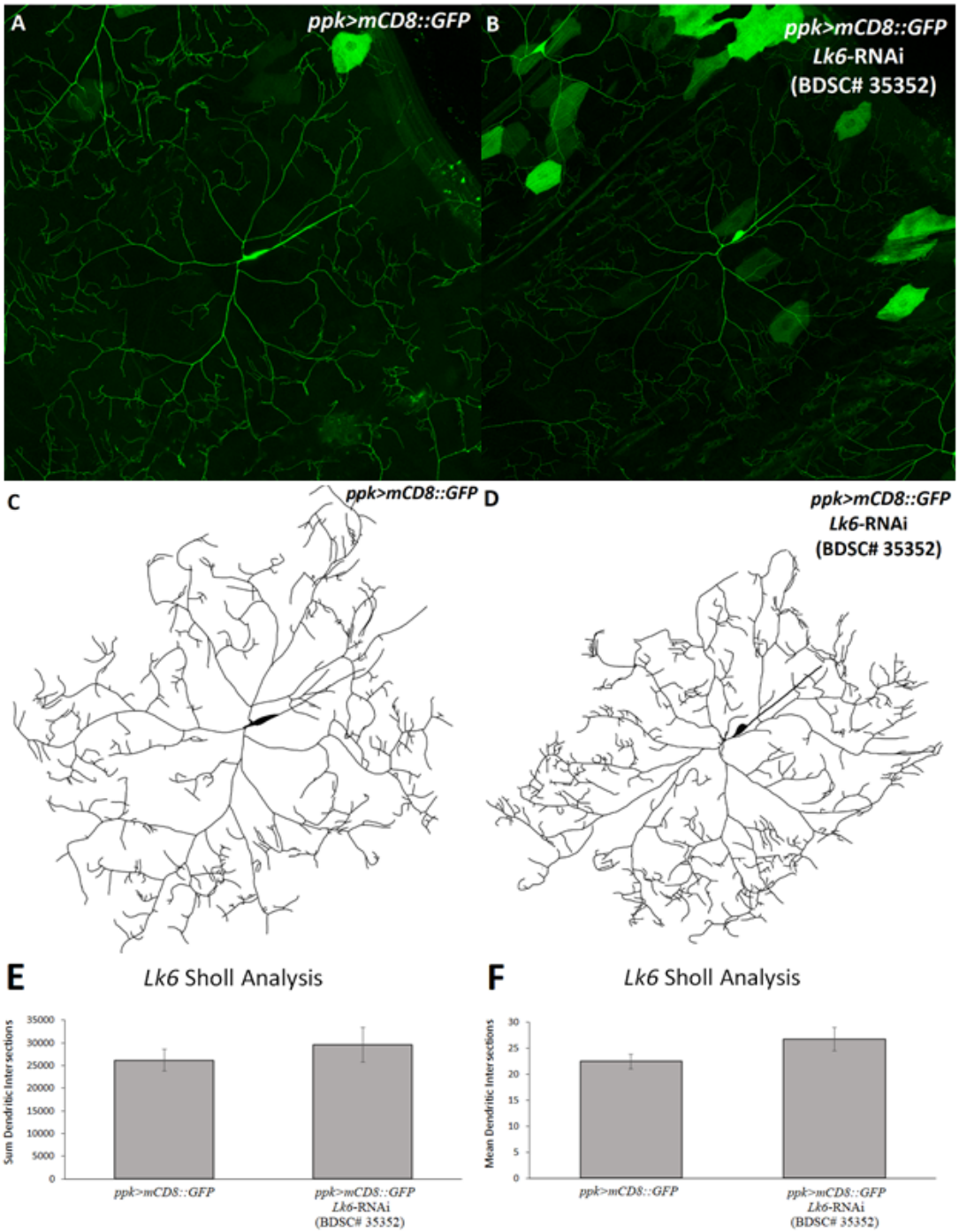

Figure 11. (A, B) Confocal micrographs displaying the dendritic arborization of class IV mdda neurons expressing *mCD8::GFP* in wild-type and *Lk6*-RNAi. (C, D) Tracings of dendritic arborizations of confocal micrographs displaying the dendritic arborization of class IV mdda neurons expressing *mCD8::GFP* in wild-type and *Lk6*-RNAi. (E, F) Sholl analysis of dendritic arborization of class IV mdda neurons expressing *mCD8::GFP* in wild-type and *Lk6*-RNAi. Nociceptor-specific knockdown of *Lk6* had no effect on larval dendritic complexity. No significant difference was seen in the total dendritic intersections or the average dendritic intersections of larvae with nociceptor-specific knockdown of *Lk6* when compared to the arbors of wild-type class IV mdda neurons.

Tor knockdown may result in increased baseline thermal nociceptor sensitivity

To examine the role of *Tor* in baseline thermal nociceptor sensitivity, *Tor* was knocked down in *Drosophila* class IV mdda neurons using the GAL4/UAS system previously described. *Tor* knockdown larvae using RNAi transgene BDSC# 33951 had an average latency of s; *Tor* knockdown larvae using RNAi transgene BDSC# 35578 had an average latency of 5.28 s, while the GAL4 only control had an average latency of 2.85 s. Both *Tor* knockdown groups were significantly less sensitive to the noxious thermal stimulus than the GAL4 only control ($p = 0.000$). These results indicated that larvae with nociceptor-specific knockdown of *Tor* showed increased sensitivity to a noxious thermal stimulus (46°C). However, UAS-*Tor*-RNAi only controls did not behave as expected. UAS-*Tor*-RNAi/+ (BDSC# 33951) had a significantly greater response latency (6.47 s) than the GAL4 only control (p=0.000) and was not significantly different from larvae with nociceptor-specific knockdown of *Tor* using RNAi transgene BDSC# 33951 (p=0.005). UAS-*Tor*-RNAi/+ (BDSC# 35578) had a significantly greater response latency (6.67 s) than the GAL4 only control (p=0.000) and was not significantly different from larvae with nociceptor-specific knockdown of *Tor* using RNAi transgene BDSC# 35578 (p=0.016). Both experimental groups and all three negative controls had a significantly lower latency than the positive, UAS-*para*-RNAi, control which had an average latency of 11 s. In this experiment a non-parametric Mann-Whitney U statistical test with an α value of 0.025 was used. Trends show that larvae expressing the *Tor*-RNAi transgene are less sensitive to noxious thermal stimuli. (Figure 12)

In attempt to gain insight into nociceptor-specific knockdown of *Tor* on baseline thermal nociception, the experiment was repeated using a different transgenic *Tor*-RNAi line

(BDSC# 34639). *Tor* knockdown larvae using RNAi transgene BDSC# 34639 had an average latency of 4.14 s. *Tor* knockdown larvae using RNAi transgene BDSC# 34639 did not show any significant differences when compared to the GAL4 only control which had an average response latency of 3.10 s (p=0.334) or the UAS-*Tor*-RNAi only control which has an average response latency of 4.44 s (p=0.068). The experimental group and both negative controls had a significantly lower latency than the positive, UAS-*para*-RNAi, control which had an average latency of 11 s. In this experiment a non-parametric Mann-Whitney U statistical test with an α value of 0.05 was used. The trends in this experiment do not align with other experiments using UAS-*Tor*-RNAi transgenes making it difficult to interpret the results of the experiments altogether. This experiment was unable to confirm the results generated using RNAi transgene BDSC# 33951 or RNAi transgene BDSC# 35578. (Figure 13)

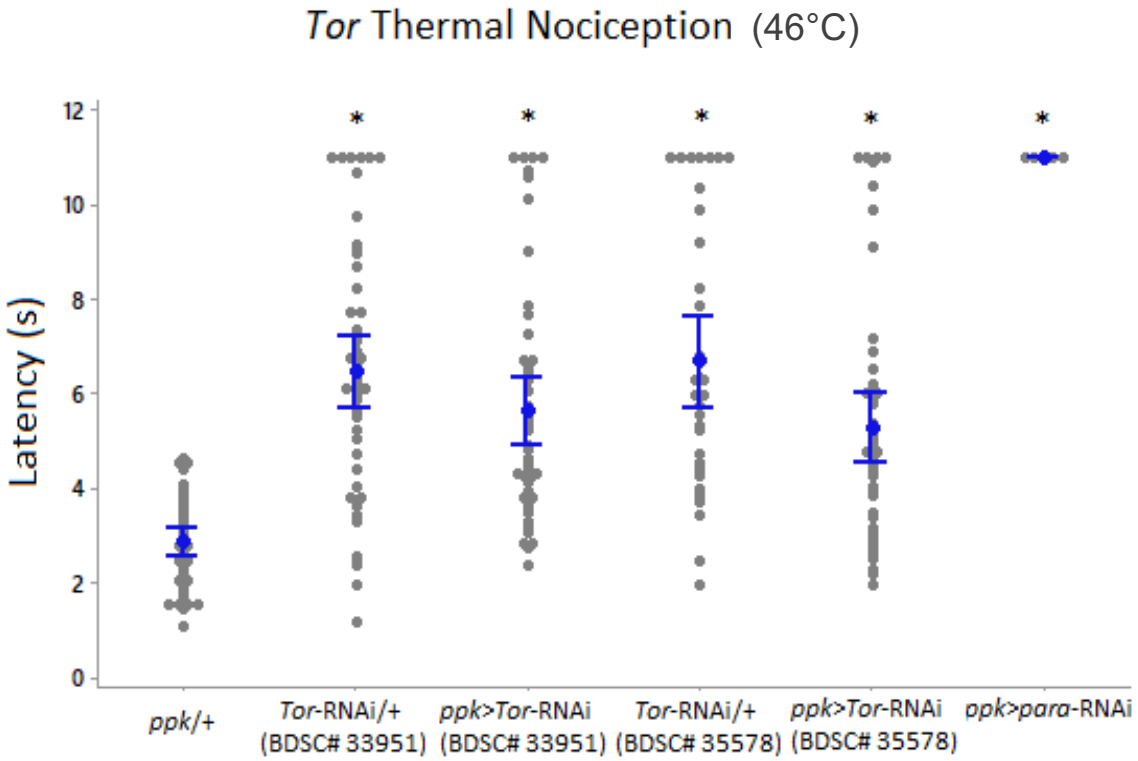

Figure 12. Larvae with nociceptor-specific knockdown of *Tor* showed significantly increased sensitivity (increased latency) to a noxious thermal stimulus (46°C) when compared to the GAL4 only (ppk/+) negative control. UAS-RNAi only negative controls also showed significantly increased sensitivity (increased latency) to a noxious thermal stimulus when compared to the GAL4 only negative control. Larvae with nociceptor-specific knockdown of *Tor* showed no significant difference in their response latency to a noxious thermal stimulus (46°C) when compared to their respective UAS-RNAi only control. Larvae with nociceptor-specific knockdown of *para* showed severely impaired nociceptive responses (increase latency) and were used as a positive control. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as an enlarged point with an interval bar representing the standard error. (n = 50 for all groups; significance determined by non-parametric Mann-Whitney U test; α value of 0.025 was used).

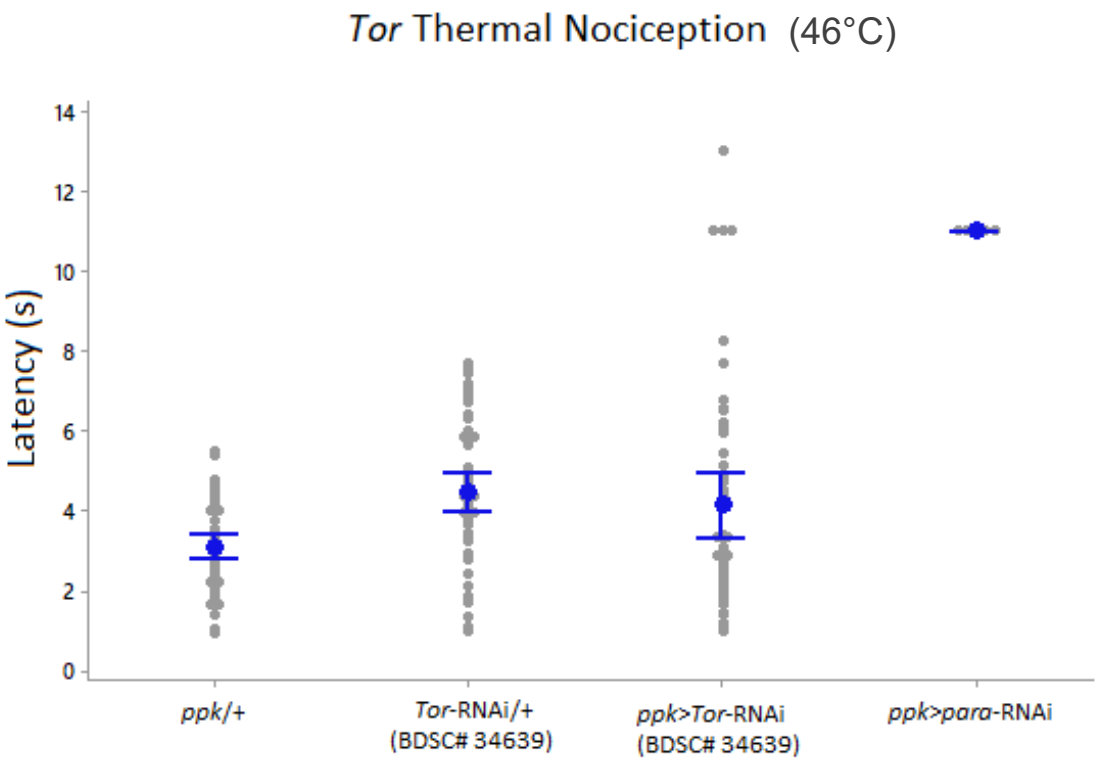

Figure 13. Larvae with nociceptor-specific knockdown of *Tor* showed no significant difference in their response latency to a noxious thermal stimulus (46°C) when compared to the GAL4 only (ppk/+) negative control. Larvae with nociceptor-specific knockdown of *Tor* showed no significant difference in response latency to a noxious thermal stimulus (46°C) when compared to the respective UAS-RNAi only negative control. Larvae with nociceptor-specific knockdown of *para* showed severely impaired nociceptive responses (increase latency) and were used as a positive control. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as an enlarged point with an interval bar representing the standard error. (n = 50 for all groups; significance determined by non-parametric Mann-Whitney U test; α value of 0.05 was used).

To examine the role of *Tor* in baseline mechanical nociceptor sensitivity, *Tor* was knocked down in *Drosophila* class IV mdda neurons using the GAL4/UAS system previously described. *Tor* knockdown larvae using RNAi transgene BDSC# 33951 resulted in 50% of the larvae responding to the noxious mechanical stimuli, *Tor* knockdown larvae using RNAi transgene BDSC# 33951 resulted in 33% of the larvae responding to the noxious mechanical stimuli, and the GAL4 only control resulted in 51% of the larvae responding to the noxious mechanical stimuli. It was found that *Tor* knockdown larvae using RNAi transgene BDSC# 33951 were not significantly different from the GAL4 only control (p=0.888), while the *Tor* knockdown larvae using RNAi transgene BDSC# 35578 were (p=0.010). (Figure 14)

In addition, when UAS-*Tor*-RNAi only controls were compared with both their respective experimental *Tor* knockdown group and the GAL4 only control in the baseline mechanical assay, a significant decrease in % response was seen. UAS-*Tor*-RNAi/+ (BDSC# 33951) resulted in 11% of the larvae responding to the noxious mechanical stimuli, and had a significantly decreased response when compared to *Tor* knockdown larvae using RNAi transgene BDSC# 33951 and the GAL4 only control ($p = 0.000$). Similarly, UAS-*Tor*-RNAi/+ (BDSC# 35578) resulted in 9% of the larvae responding to the noxious mechanical stimuli, and had a significantly decreased response when compared to *Tor* knockdown larvae using RNAi transgene BDSC# 35578 and the GAL4 only control ($p = 0.000$). This result was unexpected. If the GAL4/UAS system for cell-specific knockdown behaved as expected, the UAS-*Tor*-RNAi only controls would not be significantly different than the GAL4 only negative controls. Larvae with nociceptor-specific knockdown of *Tor* showed inconsistent responses when observing their ability to detect a noxious mechanical stimulus (50 mN) in

comparison to the GAL4 only negative control. Further experimentation must be done to determine if *Tor* plays a role in class IV mdda neurons for baseline mechanical sensitivity. (Figure 14)

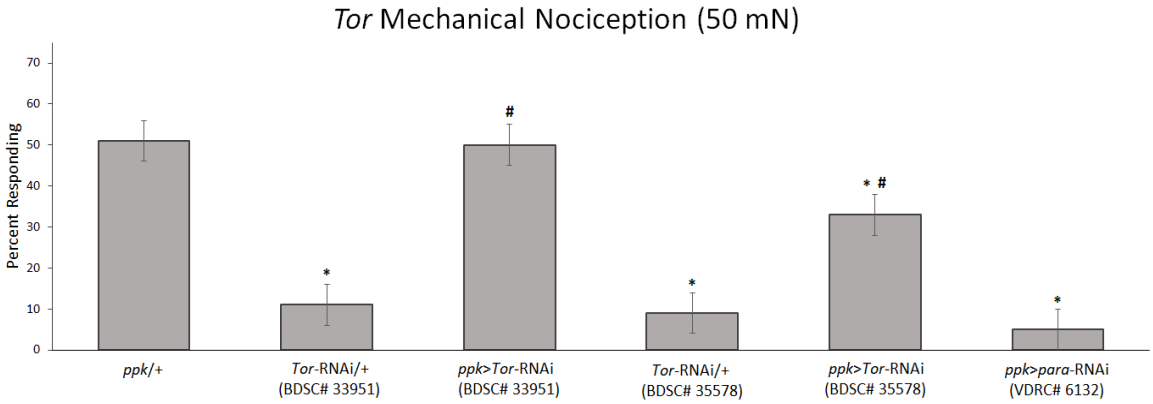

Figure 14. No conclusive trends in behavioral response are seen in larvae with nociceptor-specific knockdown of *Tor*. UAS-RNAi only negative controls showed significantly impaired baseline behavioral responses to mechanical stimuli as did knockdown of BDSC# 35578 when compared to the GAL4 only (ppk/+) negative control. Larvae with nociceptor-specific knockdown of *para* also showed a very low rate of nociceptive responses and were used as a positive control. (n = 100 for all groups; * indicates a significant difference when compared to the GAL4 only negative control; # indicates a significant difference when compared to respective UAS-RNAi only negative controls. $p < 0.025$ by Chi-Square Test)

Tor knockdown is associated with a slight increase in dendritic complexity

Because defects in nociception can potentially arise from defects in morphological development of class IV mdda neurons, it was important to identify whether nociceptor-specific knockdown of *Tor* was associated with any defects in dendritic complexity. To determine if defects in nociceptor plasticity in *Tor* knockdown larvae stemmed from developmental defects, the expression of *GFP* and *Tor* knockdown in class IV neurons was driven by *ppk1.9-GAL4>mCD8::GFP;*UAS-*dicer 2*. Live cell confocal microscopy images (Figure 15A/B) were prepared in photoshop and analyzed using Sholl analysis. Sholl analysis provides quantitative information regarding the complexity of dendrites through methods involving the measurement of interaction of dendrites with a series of concentric circles. (Figure 15C/D)

Control (*y^1 v^1; P{$y^{+t7.7}$=CaryP}attP2*) larvae expressing GFP had an average of 29,350.2 total dendritic interactions while *Tor* knockdown larva (BDSC# 33951) had an average of 33,338.8 total dendritic interactions ($p = 0.121$). (Figure 15E) There was no significant difference in the total number of dendritic interactions, however when the mean dendritic interactions were compared, it was discovered that larvae with nociceptor-specific knockdown of *Tor* showed increased dendritic complexity when looking at the mean dendritic interactions. Control (*y^1 v^1; P{$y^{+t7.7}$=CaryP}attP2*) larvae expressing GFP had an average of 21.64 mean dendritic interactions while *Tor* knockdown larva (BDSC# 33951) has an average of 26.12 mean dendritic interactions ($p = 0.018$). Significance determined using a paired *t*-test revealed that nociceptor-specific knockdown of *Tor* was associated with a slight increase in dendritic complexity and may play a role in the development of class IV nociceptive neurons. (Figure 15F)

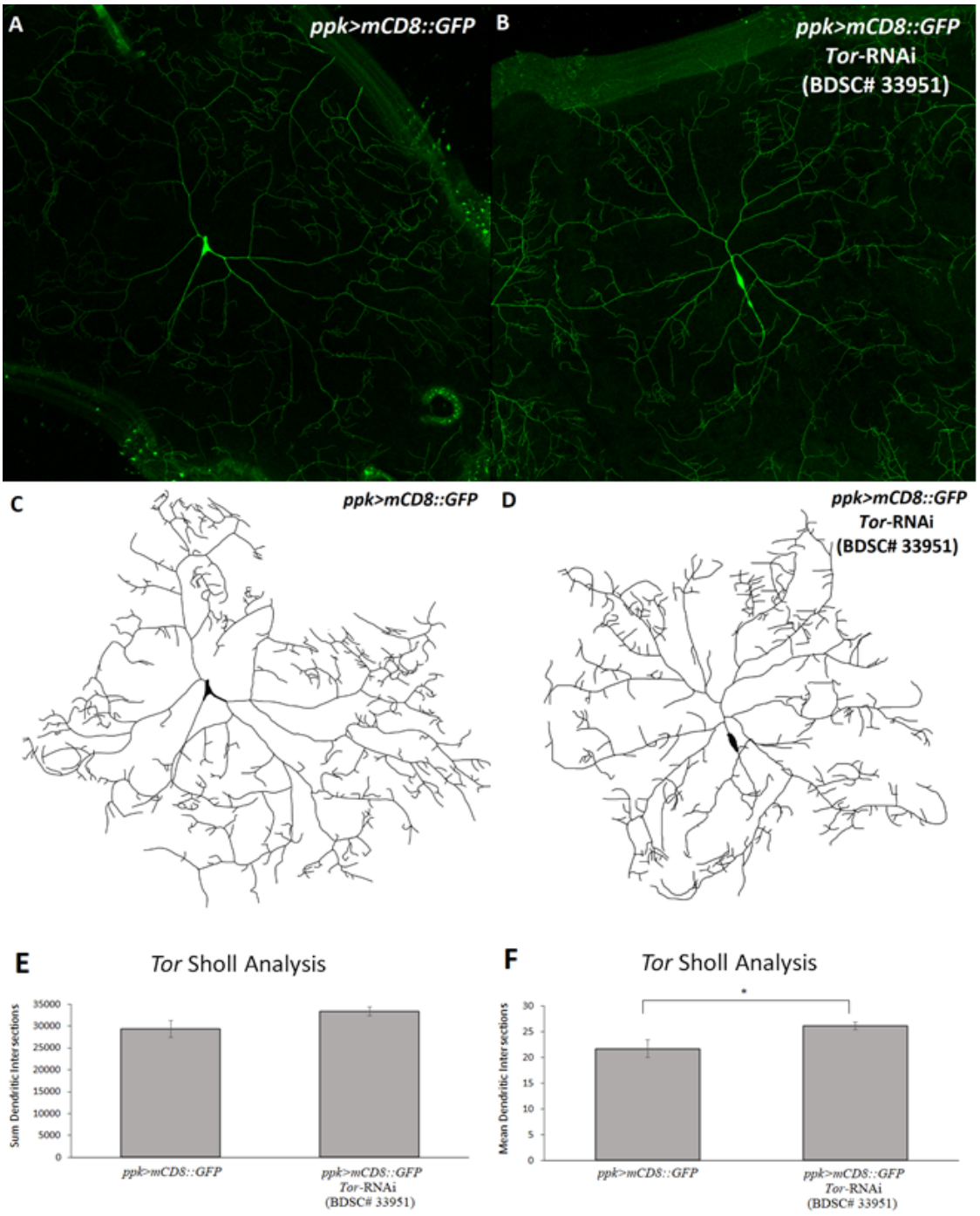

Figure 15. (A, B) Confocal micrographs displaying the dendritic arborization of class IV mdda neurons expressing *mCD8::GFP* in wild-type and *Tor*-RNAi. (C, D) Tracings of dendritic arborizations of confocal micrographs displaying the dendritic arborization of class IV mdda neurons expressing *mCD8::GFP* in wild-type and *Tor*-RNAi. (E, F) Sholl analysis of dendritic arborization of class IV mdda neurons expressing *mCD8::GFP* in wild-type and *Tor*-RNAi. Larvae with nociceptor-specific knockdown of *Tor* showed a small increase in dendritic complexity. No significant difference was seen in the total dendritic intersections of larvae with nociceptor-specific knockdown of *Tor* while they did display a significant increase in the average number of dendritic intersections when compared to the arbors of wild-type class IV mdda neurons.

Discussion

The activity-dependent regulation of protein synthesis is a core mechanism mediating neuronal plasticity. In the nociceptive system, it has been demonstrated that translation regulation pathways contribute to both the development and maintenance of chronic pain, which suggests that targeting these pathways may lead to new therapeutic treatments for chronic pain. Though synaptic plasticity is initially mediated by changes in protein trafficking, translation of synaptic mRNAs is required for structural changes in the synapse and maintenance of altered neurotransmission. [92] Protein synthesis is primarily regulated via translation initiation, which requires the binding of eIF4E to the 5' cap of the mRNA. [93] Binding of eIF4E to the 5' cap recruits eIF4G to eIF4E in a process that facilitates ribosome binding. eIF4G provides a scaffold for the binding of eIF4A to form the eIF4F complex. [94] MNK and mTOR are able to regulate translation initiation by influencing the capacity of eIF4E to bind to the 5' cap. [95] Different translation regulation targets have been shown to play very unique roles in controlling different aspects of pain sensitivity. For example, loss of 4E-BP1 leads to changes in the availability of cell-adhesion molecule, neuroligin 1, resulting in changes in mechanical sensitivity [96], while a mutation in eIF2α that inhibits phosphorylation results in changes in baseline thermal nociception with no changes in mechanical sensitivity [97]. Collectively, these studies suggest that individual translation regulation pathways may target specific subsets of mRNAs that have profound impacts on certain aspects of nociception. Translation regulation pathways that include the upstream signaling factors, mTOR and MNK, have been shown to play a role in nociceptive plasticity using both biochemical and pharmacological measures. [17, 31, 98]

In order to establish a model to study the impact of *Lk6* and *Tor* expression on nociception, the GAL4/UAS system was used to knockdown each kinase in the nociceptors. Because Lk6 and Tor use different mechanisms to influence eIF4E availability in translation initiation, it was hypothesized that each kinase would be important in controlling different aspects of nociceptor sensitivity. It was hypothesized that knockdown of *Tor* would result in decreased baseline nociceptor sensitivity and decreased morphological complexity of nociceptors. [21, 99] It was hypothesized that knockdown of *Lk6* would have no effect on baseline sensitivity or morphological complexity in nociceptors, while development of tissue injury induced hypersensitivity would be affected. [17, 28, 31, 32, 100]

Knockdown of *Lk6* resulted in phenotypic changes that supported the proposed hypothesis. Knockdown of *Lk6* had no effect on baseline thermal nociceptor sensitivity. Knockdown of *Lk6* appeared to have no effect on baseline mechanical nociceptor sensitivity, however, due to differences between the UAS-*Lk6*-RNAi negative controls and GAL4 only controls further experimentation must be done to confirm the role of *Lk6* in mechanical nociceptor sensitivity. Knockdown of *Lk6* had no effect on nociceptor morphology. And lastly, knockdown of *Lk6* was able to mitigate the effects of hypersensitization following UV-induced tissue damage. These results suggest the probable conclusion that Lk6 function is not needed for nociceptor growth or baseline function, but it may have an important role in regulation of protein synthesis during hypersensitization. This suggest that specific mRNA transcripts are being more highly regulated by Lk6 than others. It is probable that the mRNA transcripts whose translation plays a role in hypersensitization and are affected by Lk6 fall in the category of eIF4E sensitive mRNAs. (Table 2)

The finding that knockdown of *Tor* had decreased baseline thermal nociceptor sensitivity (thermal hyposensitivity) aligned with the proposed hypothesis, however, unexpected decreases in the sensitivity of the UAS-*Tor*-RNAi negative control compared to the GAL4-only control indicated that the hyposensitive phenotype could not be confidently attributed to a cell-specific knockdown of *Tor* in the nociceptors. It was also unexpected that knockdown of *Tor* resulted in a slight increase in morphological complexity of the dendrites in class IV mdda neurons. This knockdown morphological result could be confirmed through additional controls such as an UAS-*Tor*-RNAi negative control to ensure that the morphological defects can be attributed to nociceptor-specific knock down of Tor. Knockdown of *Tor* illuminated no conclusive trends in baseline mechanical nociceptor sensitivity due to significant difference in UAS-*Tor*-RNAi negative control and GAL4 only negative controls indicating that further experimentation must be done to confirm these results. These results suggest the probable conclusion that Tor plays a role in baseline thermal nociception function, and likely has an important role in regulating the expression of genes required in thermal nociception at a baseline level and perhaps in cases of hypersensitization. (Table 2)

Table 2. Summary of experimental outcomes of thermal and mechanical baseline nociception, UV-hypersensitization, and confocal microscopy assays (N/A indicates no experiment done) (BDSC- Bloomington *Drosophila* Stock Center).

Gene	RNAi BDSC#	Thermal Phenotype	Mechanical Phenotype	Hypersensitivity Phenotype	Morphological Phenotype
Lk6	35352	No Change	No Change (UAS only control inconclusive)	Hypersensitization Diminished (UAS only control inconclusive)	No Change
Lk6	28357	No Change	No Change (UAS only control inconclusive)	Hypersensitization Diminished	N/A
Tor	33951	Hyposensitive	Inconclusive	N/A	Increased Complexity
Tor	35578	Hyposensitive	Inconclusive	N/A	N/A
Tor	34639	No Change	N/A	N/A	N/A

Lk6 Does Not Affect Basal Translation of Proteins Involved in Nociception

The *Drosophila* genome contains a single *MNK* homolog, *Lk6*. [101] Lk6 is activated through phosphorylation by ERK but not by p38 MAPK signaling. Lk6, when activated, phosphorylates eIF4E at Ser251 [101], which is analogous to Ser209 in mammals, the residue that is phosphorylated by MNK. [102] The exact mechanism by which the phosphorylation of eIF4E enhances translation is not well developed. Early studies depicted that phosphorylated eIF4E has an enhanced binding affinity for the 5' cap [103], while other studies were able to show MNK-induced phosphorylation of eIF4E reduced binding affinity for the 5' cap. Reduced binding affinity may result in the disruption of the interaction between eIF4E and the 5' cap, which in turn would increase translation rates by increasing the availability of eIF4E in order to enable addition rounds of translation initiation. Though both mechanisms would act to positively enhance translation rates through, it has been found that *MNK* is not necessary for basal rates of cap-dependent translation to occur. [104]

Though there are limited studies regarding the role of *Lk6* in baseline nociceptor sensitivity, many mammalian studies have focused on the role of *MNK* in a post-injury scenario. In most studies, mammals with a mutation in Ser209, the *MNK* phosphorylation site on eIF4E, or mutations resulting in complete loss of function of *MNK* were used to decrease the function or availability of MNK proteins. In these studies, it was found that there was no difference between mutants and controls when looking at baseline thermal and mechanical nociceptor sensitivity [31, 98]. This information led the hypothesis that *MNK* does not play a role in baseline thermal or mechanical nociceptor sensitivity. Evidence gathered from the research conducted supports the hypothesis that phosphorylation of eIF4E by *Lk6* is not

required for baseline thermal or mechanical nociception and the conclusion that regulation of translation through *Lk6* has no effect on baseline nociceptor sensitivity.

To provide further evidence for the role of *Lk6* in baseline nociceptor function, morphological analysis of dendrites lacking *Lk6* in the class IV mdda neurons was done. It was found that larvae lacking the eIF4E kinase, Lk6, successfully developed dendritic arbors of normal complexity. Previous studies have shown conflicting data regarding the role of eIF4E phosphorylating kinases in the development of both insects and mammals. In mice, it has been shown that knockout of *MNK1*, *MNK2* or both resulted in offspring that were viable, fertile, and developed normally, indicating that eIF4E phosphorylation is not essential for growth and development. [105] However, studies in *Drosophila* provide evidence that phosphorylation of eIF4E by Lk6 is vital for development. Mutation of ser251, the Lk6 phosphorylation site on eIF4E, in *Drosophila* resulted in reduced viability, slower development, and reduced adult size, demonstrating that phosphorylation of eIF4E is required for normal organismal growth. [106] In this study, it was hypothesized that *Lk6* is not involved in the development of nociceptive neurons as they appeared to function normally in the behavioral assays for baseline nociception. The findings support the hypothesis. It was found that no developmental defects were found in the class IV mdda of *Drosophila* larvae with nociceptor-specific knockdown of *Lk6.* These findings align with mammalian studies that indicate eIF4E phosphorylation is not essential for growth and development, however, because the knockdown of Lk6 was limited to nociceptors, developmental defects caused by genome-wide loss of *Lk6* are still probable.

Evidence from morphological analysis and baseline behavioral nociception assay indicate that *Lk6* does not cause any significant changes in translation of proteins that are necessary for normal nociception from both the molecular and structural standpoint.

Lk6 Regulates Synthesis of Proteins Required for Nociceptor Sensitization

To further investigate the role of *Lk6* in translation regarding nociception, an injury-induced hypersensitization model using UV radiation was used to determine if *Lk6* regulates synthesis of proteins required for neural plasticity. The proper localization and synthesis of proteins is essential for synaptic plasticity. Synaptic plasticity requires the translation of proteins that modulate synapse function. [107] Although synaptic plasticity is initially mediated by changes in protein trafficking, translation of synaptic mRNAs is required for structural changes to the synapse and to maintain states of chronic pain. [92] Synaptic translation initiation is believed to be mediated via *mTOR* and *MNK* signaling pathways, downstream of *ERK*.

Many studies are in agreement that both *mTOR* and *MNK* are required for nociceptor hypersensitivity following tissue damage. Studies in mice have developed a compelling case for *MNK* signaling as a core signaling pathway in the generation of thermal and mechanical hypersensitivity as well as ongoing pain in response to inflammation. [31, 53] Mice that have a mutation at the MNK phosphorylation site on eIF4E show have defective development of nociceptive behavioral plasticity in response to inflammatory mediators such as NGF and fail to show increased nociceptor excitability in response to these mediators. [98] The experiment done in this study, on *Drosophila* larvae with nociceptor-specific knockdown of *Lk6,* show conclusive results that knockdown of *Lk6* was able to stop the development of thermal

hypersensitivity after injury induced by UV radiation. This signaling mechanism has been show to function similarly in studies of chronic pain development in both the fly and mouse models.

This model can be used to help understand the transition from acute to chronic pain, where blocking eIF4E phosphorylation could be a primary target in blocking the transition to a chronic pain state after injury. In mammals, phosphorylation of eIF4E by MNK is known to regulate the translation of a number of mRNA transcripts that are involved in nociceptive plasticity. These mRNA transcripts include matrix metalloprotease 9 (MMP9) which has shown to be upregulated in cases of autism causing alterations in synaptic plasticity and dendritic spine morphogenesis, [108] brain-derived neurotrophic factor (BDNF) which has been shown to regulate mediate hyperalgesic priming in the mammalian dorsal root ganglion (DRF) [100], and many cytokines and chemokines that are involved in pain signaling. Similar results have been shown in *Drosophila*, where the Lk6-eIF4E signaling pathway has been shown to modulate the availability of molecules necessary for sensitization, including molecules that modulation neural transmission such as glutamate and its receptors. [18, 52]

Another potential translational target of the MNK-eIF4E signaling pathway is glutamate receptor, GluRIIA. Glutamate is known to mediate the majority of the neurotransmission in the CNS in mammals. Following the establishment of a synapse, its strength can be modified by changes in synaptic size, presynaptic glutamate release, and the number of post synaptic glutamate receptors. The capacity of the synapse to change during nociceptor plasticity requires regulated expression of glutamate receptors, such as GluRIIA, by pathways such as the MNK-eIF4E signaling pathway to modulate synapse strength. Previous studies done in *Drosophila* show that *Lk6,* the *Drosophila* homolog of *MNK*, is

required proper localization of GluRIIA in both pre and post synaptic neurons. *Lk6* likely signals through eIF4E to regulate the synaptic levels of GluRIIA. It has been shown that disrupting the binding of eIF4E to eIF4G or inducing the expression of a non-phosphorylatable isoform of eIF4E results in a significant reduction in GluRIIA at the synapse and this reduced neural transmission. This evidence suggests that proper expression of GluRIIA is necessary for proper neural transmission and its expression is potentially regulated by the Lk6-eIF4E signaling pathway during the development of neural plasticity. However, the role of glutamate in *Drosophila* nociception has yet to be discovered. [18]

A hallmark of peripheral sensitization is increased thermal sensitivity which is known to be supported by ERK (the molecule known to phosphorylate and activate MNK) activation and subsequent synthesis of proteins and ion channels necessary for nociception. [87] Studies in mice have shown that pharmacologic inhibition and genetic inactivation of ERK disrupted the function of TMEM16A. TMEM16A is a receptor-activated calcium-dependent chloride channel that is analogous to anoctamin 1 (ANO1). [109] It has been shown that interaction between ANO1 and TRPV1, a thermally sensitive calcium channel, causes strengthened action potentials that result in strong pain sensations. Peripheral sensitization is commonly understood to be mitigated by increased thermal sensitivity which is thought to be supported by ERK activation a subsequent synthesis of the TRPV1 receptor and its transport to the axon terminals in damages tissue. [17] There was also a correlation between the expression levels of Nav1.7, a sodium channel known to pay a role in nociception, ERK, and the degree of inflammatory pain. Inhibition of Nav1.7 causes a decline of ERK expression and Nav1.7 protein levels were also increased with increased phosphorylation of ERK. [110] With this information, it is evident that the ERK-MNK-eIF4E phosphorylation pathway plays a role in

the development of nociceptor and potentially by many different and complex mechanism. Because, ERK signaling to downstream effectors, *MNK* (in mammals) or *Lk6* (in *Drosophila*) has a well characterized effect of the development of hypersensitization, the hypothesis that *Lk6* will regulate the expression of factors such as GluRIIA and ion channels that are necessary for hypersensitization, and when knocked down, *Lk6* will not be able to produce hypersensitive phenotypes.

Tor May Effect Baseline Nociception and Likely Plays a Role in Hypersensitization

Tor is considered to be a vital component in positive translation regulation through control of downstream effector eIF4E via phosphorylation of 4E-BP1and has been shown to play a role in cell growth and the control of hypersensitivity following injury. mTOR has been shown to be necessary for the development of thermal hypersensitivity following tissue injury in mice. Surgical procedures, such as plantar incision, facilitate production of algogenic and/or hyperalgesic molecules, leading to peripheral sensitization. Similar to hypersensitization studies done with *MNK*, it was shown that thermal sensitivity induced by tissue-damage, in this case plantar incision, was mediated through another regulator of glutamate, vesicular glutamate transporter 2 (VGLUT2). The expression of VGLUT2 along with thermal hypersensitivity was reduced when mTOR was inhibited using rapamycin. This suggest that mTOR does play a role in the development of hypersensitization and perhaps through modulation of the expression of molecules involved in neurotransmission such as glutamate, a phenomenon also seen downstream of the MNK-eIF4E signaling axis. [111]

Mice models have shown that mTOR plays a role in proper sensory transmission of noxious stimuli. Vertebrate primary afferent nociceptors can be divided into Aδ fibers, that

are myelinated and mediate the first sensation of sharp and rapid pain, and C fibers that are unmyelinated and sense secondary delayed or diffused pain. [17] Studies utilizing the mTOR inhibitor, rapamycin, showed reduced sensitivity that acted in part by reducing the sensitivity of Aδ fibers that are responsible for the "first" or fast pain produced by a noxious thermal stimulus. Inhibition of mTOR did change the electrical excitability of slow transduction C fibers. [21] This suggest that *Tor* could be used to reduce the initial pain generated by a noxious stimulus through reduction in the function of Aδ fiber function, however whether this phenomenon occurs independently of inflammation or prior injury is unknown. In opposition to this hypothesis it has been shown that mTOR is not required for baseline nociceptor sensitivity. Chronic systemic treatment with CCI-779 (a rapamycin analog) inhibited the mTOR pathway in sensory axons reducing mechanical and cold hypersensitivity after peripheral nerve injury without affecting the nociceptive threshold in naive controls [87]. Conflicting and unclear conclusions regarding the role for mTOR in baseline nociception supports the need for continued research on translational control of genes involved in nociception. For the presented research, it was hypothesized that mTOR would be important in baseline nociception given its reputation of the master regulator translation initiation. [17]

In this study, it was found that nociceptor-specific knockdown of *Tor* in *Drosophila* resulted in decreased thermal nociceptor sensitivity. During the thermal assay conducted at 46℃, decreased nociceptor sensitivity, was seen not only in the experimental *Tor* knockdown groups but also in RNAi-only groups that controlled for insertion of the RNAi transgene. These results indicated that while interruption of the *Tor* gene may cause defects in the larval

ability to detect a noxious stimulus, it cannot be specifically attributed to cell-specific knockdown.

Morphological analysis of nociceptors lacking *Tor* in *Drosophila* showed a slight increase in dendritic complexity when looking at the average of dendritic intersections, however, this phenomenon was not seen when looking at the total number of dendritic intersections using Sholl analysis. This result is unexpected and cannot be fully attributed to nociceptor-specific knockdown of *Tor* given inconclusive controls found in mechanical and thermal assays. *mTOR* is known to be involved in signaling pathways that control cell growth, nutrient metabolism, and protein translation. *mTOR* regulates many functions in the development of the brain, such as proliferation, differentiation, cell migration, and dendrite formation. In addition, *mTOR* is important in synaptic formation and plasticity. Abnormalities in *mTOR* activity is linked with severe deficits in nervous system development, including tumors, autism, and seizures. *mTOR* signaling pathways are essential to neurogenesis and the establishment of neural circuits. In order to further investigate the morphological results found in this experiment, additional controls, such as a UAS-*Tor*-RNAi negative control, controlling for insertion of the RNAi transgene would be needed to attribute any changes in class UV mdda neurons to loss of function of *Tor.* [99]

Inconclusive results were found through mechanical nociception assay following nociceptor-specific knockdown of *Tor*. The experimental *Tor* knockdown group showed trends that indicated that the expression of *Tor* has no effect on baseline mechanical nociception; one experimental group did not differ from the GAL4 only control and the other varied only slightly from the GAL4 only control. Significant differences between the UAS-

Tor-RNAi only negative controls GAL4 only control and the other varied only slightly from the GAL4 only control, while groups controlling for the transgene insertion showed reduced mechanical sensitivity. The results of the mechanical experiment were, therefore, rendered inconclusive. Though the results were inconclusive, the trends align with previous literature that suggest that translation control of mTOR has no effect of nociceptors ability to detect a noxious mechanical stimulus. Studies utilizing mice models have shown that inhibition of mTOR using rapamycin showed no change in the expression of proteins, such VGLUT3, a glutamate transporter known to be required in mechanical nociception, but did show increases in VGLUT2, a glutamate transporter known to be involved in thermal nociception via expression with TRPV1, an ion channel shown to responsible for thermal signal transduction. These studies could suggest that similar mechanism could be functioning to regulate the translation of thermally sensitive *Drosophila* TRP channels such as TRPA1. [111-114]

Tor Transgenic Drosophila Lines Revealed Inconsistencies Between Negative Controls

Throughout the mechanical and thermal assays, difference between the GAL4 only and UAS-RNAi controls were seen. These negative controls are important as they control for and behavioral effects caused by individual aspects of the GAL4/UAS-RNAi system. When comparing GAL4 only controls to UAS-RNAi only controls it is expected that they will not be significantly different to one another. In order to address the problem between negative control groups within the mechanical assay of both *Tor* and *Lk6* knockdown, a revised mechanical assay could be used. Stimulation with a Von-Frey filament could remain a noxious stimulus while behavioral responses could be more robustly analyzed. In this

experiment only NEL was quantified. However, it has been shown that *Drosophila* larva exhibit multiple behavioral responses to noxious stimuli. *Drosophila* larvae typically have a distinct response to thermal nociception, a corkscrew-like roll toward or away from the stimulus. This response is typically proceeded by c-shaped body bending, however at times the body bend is sufficient for noxious stimuli relief and will not be followed by NEL; this escape method is termed "bending". [115] . Noxious mechanical stimuli have also been shown to cause head casting and head retraction in *Drosophila* larvae. [116] Behavioral screens in *Drosophila* have also identified mutations that cause a "bang-sensitive" (BS) behavioral phenotype, that is, they paralyze and seize following mechanical stimulation. One mutant was shown to exhibit neuronal hyperexcitability and paralysis for ~20 seconds when subjected to mechanical stimuli. [117, 118] This behavioral phenotype was observed but not quantified during the mechanical assay for nociceptor-specific knockdown of *Lk6.* A more robust video analysis of the behaviors exhibited by *Drosophila* larva with nociceptor-specific knockdown could perhaps illuminate conclusive effects of the genes on translation and nociception.

However, robustness of the thermal assay is not a probable cause of differences seen between negative control groups as the delivery of the stimulus and method of analysis is highly regulated. When comparing GAL4 only controls to UAS-RNAi only controls it is expected that they will not be significantly different to one another. The currently lines cannot draw conclusive results due to significant difference in the negative control groups. In order to further investigate the role of *Tor* in translation and nociception, experimental transgenic lines and their controls that act appropriately must be found or created. The differences that have been observed between GAL4 only controls and UAS-RNAi only

controls could be due to off-target effects, though the GAL4/UAS system effectively reduced the probability of off-target effects, or the insertion of the transgene could have caused defects in the chromosome. In order to move forward, transgenic lines could be ordered from Vienna *Drosophila* Resource Center (VDRC) and tested to see if groups controlling for RNAi transgene insertion would react more similarly to GAL4 only controls. Dominant-negative mutations have also been used extensively in flies with the GAL4/UAS system, an alternative to RNAi-mediated gene knockdowns to disrupt gene activity. Dominant-negative versions of the protein of interest provide an independent means to confirm that effects obtained with such molecules accurately represents loss of function phenotypes. Lastly, down steam targets could be specifically mutated in ways that mimic the loss of function of their upstream regulator. For example, lines that have mutations in 4E-BP at the phosphorylation site of Tor could be used to analyze the role of *Tor*.

Future Directions

In the future, conclusive results for *Lk6* knockdown in mechanical nociception will be found. Experiments looking at the phosphorylation status of eIF4E following knockdown of *Lk6* would be useful in confirming the role of *Lk6* in the nociceptive neurons of *Drosophila*. It would also be useful to look at general translation rates and the changes that occur following *Lk6* knockdown. Discrepancies in *Tor* transgenic lines will be elucidated and conclusive baseline mechanical and thermal nociception effects will be found, hopefully through the use of new transgenic lines obtained from the Vienna *Drosophila* Resource Center. Further, because it is hypothesized that *Tor* will play a role in both baseline nociception and the development of injury induced hypersensitization, a tissue injury induced

hypersensitization assay will be formed and the role of *Tor* in chronic pain development will be explored. Lastly, it is important that a more robust mechanical nociception assay exploring a wide array of behaviors is used for both *Tor* and *Lk6* to analyze the responses produced by noxious mechanical stimuli.

Conclusion

It can be concluded that translation of proteins is essential to the proper development and function of the *Drosophila* nervous system. Well characterized signaling pathways that include the kinases Tor and Lk6 are imperative in the process of nociception and are able to regulate translation in unique ways. It was found that nociceptor-specific knockdown of *Lk6* played an important role in the development of thermal nociceptor sensitivity following UV induced tissue injury while playing no role in baseline nociceptor sensitivity. *Lk6* likely controls the translation of mRNAs known to be involved in nociceptor plasticity or signal transduction. These mRNAs may include sodium and calcium channels as well as molecules such as GluRIIA, MMP9, chemokines, and cytokines that play a role in nociception. Nociceptor-specific knockdown of *Tor* showed phenotypes that eluded toward the involvement of the *Tor* signaling pathway in baseline nociceptor sensitivity and it hypothesized that *Tor* will play a role in the translation of mRNAs known to be involved in nociceptor plasticity in future experiments.

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Vita

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